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Mitochondrial alternative pathway is associated with development of freezing tolerance in common wheat

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Summary

Cold acclimation is an adaptive process for acquiring cold/freezing tolerance in wheat. To clarify the cultivar difference of the freezing tolerance, we compared mitochondrial respiration activity and expression profile of the alternative oxidase (AOX) genes under the low temperature condition using two common wheat cultivars differing in freezing tolerance. During cold acclimation, the respiration capacity of alternative pathway significantly increased in a freezing-tolerant cultivar compared with a freezing-sensitive cultivar. More abundant accumulation of the *AOX* and uncoupling protein gene transcripts was also observed under the low temperature conditions in the tolerant cultivar than in the sensitive cultivar. These results suggested that the mitochondrial alternative pathway might be partly associated with the cold acclimation and freezing tolerance in wheat.

Key Words: alternative oxidase, alternative pathway; cold acclimation; low temperature; *Triticum aestivum* L.

Abbreviations: AOX, alternative oxidase; COR, cold responsive; CS, Chinese Spring; LEA, late embryogenesis abundant; M808, Mironovskaya 808; SHAM, salicylhydroxamic acid; *UBI*, *ubiquitin*; *WhUCP1*, *wheat uncoupling protein 1*

Introduction

Plant mitochondria possess a unique respiratory pathway, the cyanide-insensitive and salicylhydroxamic acid (SHAM)-sensitive alternative pathway, besides the main cyanide-sensitive cytochrome pathway (Henry and Nyns, 1975). The alternative pathway is a non-phosphorylating electron transport pathway branching from the cytochrome pathway at the ubiquinone pool, and the electron flow through the pathway reduces oxygen to water without conservation of energy in the form of ATP (Lambers, 1982; Siedow, 1982). A variety of biotic and abiotic stress conditions have been shown to give a negatively impact on the cytochrome pathway and induce AOX (Vanlerberghe et al., 2002). When the capacity for the cytochrome pathway respiration is reduced, some signals lead to coordinate changes in the alternative respiration capacity (Vanlerberghe and McIntosh, 1997). Low temperature stress also increases the capacity of the alternative pathway (Vanlerberghe and McIntosh, 1992; Purvis and Shewfelt, 1993), and steady-state levels of mRNA from some of *AOX* genes increased under low temperature conditions (Ito et al., 1997; Takumi et al., 2002; Fung et al., 2006; Sugie et al., 2006). Recently, it was reported that the AOX activity plays a role in shoot acclimation to low temperature in *Arabidopsis* (Fiorani et al., 2005).

We previously isolated two non-homoeologous genes, *WAOX1a* and *WAOX1c*, encoding the AOX proteins from common wheat (Takumi et al., 2002). The two AOX genes were commonly responsive to low temperature, but they showed different responses to cyanide. WAOX1a alleviates oxidative stress when the cytochrome pathway of respiration is inhibited under low temperature stress conditions (Sugie et al., 2006). Under the low temperature condition, it is well known that a series of *COR* (cold responsive)/*LEA* (late-embryogenesis-abundant) genes are transcriptionally activated, and the accumulated COR/LEA proteins contribute to promoting the development of freezing tolerance (Thomashow, 1999). Difference of the *COR*/*LEA* gene expression patterns is related with cultivar difference of the cold/freezing tolerance in wheat (Kobayashi et al., 2004). However, there is limited information about contribution of the alternative respiration pathway and AOX expression to the cultivar difference of cold/freezing tolerance. Under drought and salt stress conditions, the *Vigna AOX2b* gene regulation was differed between tolerant and sensitive cultivars (Costa et al., 2007). Our aim of the present study is to confirm relationship between the alteration of mitochondrial respiration pathway under low temperature stress and the cultivar difference of cold/freezing tolerance. Here, we compared low temperature-responsive expression patterns of *WAOX1a* and *WAOX1c* and alternative pathway capacities in two wheat cultivars showing distinct levels of freezing tolerance.

Materials and methods

A winter cultivar 'Mironovskaya 808' (abbreviated as M808) and a spring cultivar 'Chinese Spring' (CS) of common wheat (*Triticum aestivum* L.) were used as a cold/freezing tolerant and susceptible accessions, respectively (Ohno et al., 2001). Seeds of M808 and CS were planted in pots with soil, and were grown in a growth cabinet at 22˚C with a 16 h photoperiod (the standard condition).

For estimation of respiration activity, wheat seedlings were grown under the standard condition for 2 weeks and then transferred to 4˚C. The leaves were dissected using a scalpel into appropriate sizes (50 mg fresh weight) and vacuum infiltrated with the buffer solution (0.1 M Tris-HCl (pH 9.5) and 1 mM phenylmethylsulfonyl fluoride) for 2-3 min to allow penetration of oxygen and inhibitors into the tissues. The capacity of the cytochrome pathway was estimated by measuring the rate of oxygen uptake inhibited by 0.4 mM antimycin A in the presence of 0.4 mM SHAM, while that of the alternative pathway was estimated by measuring the rate of oxygen uptake inhibited by 0.4 mM SHAM in the presence of 0.4 mM antimycin A. The rate of oxygen uptake was measured using a Clark type oxygen electrode (Rank Brothers, Cambridge, UK) in the buffer, to which the same concentrations of inhibitors as those added during growth were added prior to the measurement, according to Wagner and Wagner (1997). Means with standard error were calculated based on 5 independent experiments.

For studies of gene expression, 7-day-old seedlings of CS and M808 grown under the standard condition were transferred to 4˚C. Total RNA was extracted from the seedling leaves and treated with DNaseI to remove the contaminated DNA. Reverse transcriptase (RT)-PCR with the gene-specific primer sets resulted in amplification of the specific single fragments as previously described (Sugie et al., 2006; 2007). RT-PCR for *WhUCP1* was conducted with the following primers; 5'-TGAAAGTGAGATTGCAAGCA-3' and 5'-GCCCATCATTCTTGATTTCA-3'. Advantage 2 polymerase mix (BD Bioscience) was exceptionally used only for the *WAOX1a* and *WAOX1c* amplification. The amplification at fewer cycles for each gene was in the exponential range of amplification. For RT-PCR analysis of the *WAOX1a* and *WAOX1c* expression, 28 and 32 amplification cycles were in the exponential range, respectively. As an internal control, fragments from wheat ubiquitin gene (*UBI*) were amplified (Sugie et al., 2006). Quantitative RT-PCR was performed using a Line Gene Fluorescent Quantitative Detection System (Bio Flux, Tokyo, Japan) and gene specific primer sets. As an internal control, *UBI* was used. The rate of amplification was monitored by

SYBR® Green Real-time PCR Master Mix (TOYOBO, Osaka, Japan) according to the manufacture's protocol. Results were obtained as $2^{-\Delta Ct}$, where ΔCt is the number of PCR cycles required to the log phase of amplification for *WAOX1a* minus the same measure for *UBI*, and then were represented as relative values to the *WAOX1a* transcript level in CS under the standard condition.

Immunoblot analysis was performed using a total protein fraction prepared from seedlings grown either under the standard condition (22˚C) or under the low temperature condition (4˚C). For the mitochondrial protein isolation, leaf tissues (0.3 g) were chopped in 0.9 mL of extraction buffer containing 50 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid and 10 mM 2-(N-morpholino) ethanesulfonic acid (pH-6.6) on ice. After three times of centrifugation at 15,000-x g for 10 min at 4˚C, the supernatant was used for SDS-polyacrylamide gel electrophoresis. Soluble proteins (15 μg) were resolved through 12.5% polyacrylamide gel and electro-transferred onto nitrocellulose membranes, Hybond-C Extra (Amersham Biosciences, Piscataway, USA). After incubation with specific antibodies, protein blots were developed using a Vectastain universal ABC kit (Vector laboratories, Burlingame, California, USA) as described by the manufacturer. Monoclonal antibody against AOX (Elthon et al., 1989) was a gift from T. Elthon (University of Nebraska, USA). Intensity of the AOX bands was assessed by scanning the blots with NIH IMAGE 1.61 software, and the relative values were calculated after normalized by the CBB stains.

Results and discussion

M808 was bred in Mironovskaya Institute, Ukraine, and reported to be the hardiest winter cultivar among tetraploid and hexaploid wheat tested for freezing tolerance (Veisz and Sutka, 1990). Our previous studies also showed the much higher freezing tolerance of M808 compared with that of CS by the simple one-point assay (Ohno et al., 2001; Kume et al., 2005). It has been demonstrated that such cultivar difference of freezing tolerance could be at least partly caused by the differential accumulation levels of *COR*/*LEA* transcripts during cold acclimation (Vágújfalvi et al., 2000; Kobayashi et al., 2004). In the present study, we found the other factors affecting the cultivar difference of cold/freezing tolerance in wheat.

Respiration activities/capacities were compared in seedling leaves under low temperature conditions between CS and M808. No difference was observed in the rate of total O_2 consumption under the standard condition in the two cultivars. The rate gradually increased until day 10 under low temperature in both cultivars. M808 showed slightly more total O_2 consumption rate than CS under the low temperature conditions, but no significant difference

was observed in the two cultivars (Fig. 1A). The cytochrome pathway accounted for a major part of the total respiration activity under the standard condition, and the level of its capacity was equivalent in CS and M808 (Fig. 1B). The capacities of the cytochrome pathway greatly decreased within 1 d under low temperature in both cultivars. The total O_2 consumption rate in the cytochrome pathway of CS was fully recovered within 3 d of low temperature stress, while in M808 it was recovered up to the about 1.3-times level of the standard condition after 3 d low temperature. On the other hand, the capacity of the alternative pathway significantly increased within 1 d under low temperature, and remained at high levels during the low temperature conditions in M808. No significant change, however, was observed in the capacity of the alternative pathway of CS. The ratio of the alternative pathway to the cytochrome pathway obviously increased within 1 d under low temperature in both cultivars (Fig. 1C). The significantly higher ratio in M808 than in CS after 7-d low temperature treatment was apparently due to the increased alternative pathway capacity. These results indicated that the alternative pathway activity enhanced up to the higher levels under the low temperature conditions in M808 than in CS.

In *Arabidopsis*, it was previously reported that low temperature reduces the transcript accumulation levels of some mitochondrial genes including *COX6b* and *ATP9*, coupled with the reduction of the cytochrome pathway capacity (Sugie et al., 2006). However, transcript accumulation levels of three mitochondrial genes, i.e., *NAD3*, *COXI* and *ATPA*, did not show any significant changes in both wheat cultivars under the low temperature conditions (data not shown). Similarly, no significant changes were observed in mitochondrial ribosomal protein genes, *TaMRPL5* (Sandoval et al., 2004), *TaMRPL11* (Handa et al., 2001) and *WHLP* (Mizumoto et al., 2004). In these genes, cultivar differences were also not significant throughout the low temperature treatment (data not shown).

A transcript level of *WAOX1a* showed a steady increase until the 5-d under low temperature, agreeing with our previous result (Fig. 2A; Takumi et al., 2002). The *WAOX1a* transcript showed gradual accumulation in the leaves and the level reached high plateaus by 5 days in CS. Thereafter, the *WAOX1a* transcript decreased in CS. M808 accumulated slightly higher levels of the *WAOX1a* transcript under the low temperature conditions than CS and remained at high levels toward day 10 (Fig. 2B). About twice amount of the *WAOX1a* transcript was accumulated in M808 than in CS after 10-d low temperature treatment (Fig. 2C). A transcript level of *WAOX1c* also showed a steady increase in M808 until day 5 under low temperature, and M808 accumulated slightly higher levels of the *WAOX1c* transcript than CS. No significant changes of the AOX levels were observed within day 5 under low temperature between the two cultivars. Thereafter, the protein accumulation levels of AOX

gradually increased in M808 but decreased in CS under the low temperature conditions (Fig. 3). The AOX levels were significantly higher in M808 than in CS after 7-d low temperature treatment.

During cold acclimation, the respiration capacity of alternative pathway significantly increased in M808 (Fig. 1). The higher levels of the alternative pathway capacity in M808 seemed to be coupled with the abundant accumulation of the *AOX* transcripts (Fig. 2). It should be concluded that the high levels of the *AOX* transcript accumulation and the alternative pathway activity at least partly contributed on development of the cold/freezing tolerance in M808. Our previous study also showed that overexpression of the *WAOX1a* gene practically reduced production of reactive oxygen species under the low temperature condition in transgenic *Arabidopsis* (Sugie et al., 2006). Recently, it was reported that the altered levels of AOX protein resulted in altered leaf growth phenotypes under low temperature condition in transgenic *Arabidopsis* plants (Fiorani et al., 2005). Together with these previous studies, our present observation suggested that one of genetic factors determining the cultivar difference of cold/freezing tolerance is responsibility of the alternative pathway to the low temperature condition in wheat. Dramatic increase of wheat *COR*/*LEA* genes was previously observed within 1-d treatment of low temperature (Kobayashi et al., 2004), and the *COR*/*LEA* expression patterns were not corresponding to the gradually increasing pattern of the *AOX* transcript accumulation, suggesting that the responsibility might be associated with development of the cold/freezing tolerance independently of the *COR*/*LEA* regulation pathways. Wheat *COR*/*LEA* expression is mainly controlled by the *Fr-1* major gene (Kobayashi et al., 2005), and therefore, the mitochondrial alternative pathway seems to contribute on development of the cold/freezing tolerance independently of *Fr-1* in common wheat.

It was also reported that a transcript accumulation level of mitochondrial uncoupling protein (UCP) gene was increased by exposure to low temperature in potato and *Arabidopsis* (Laloi et al., 1997; Maia et al., 1998). UCP dissipates the proton electrochemical gradient across the inner membrane to produce heat instead of synthesizing ATP (Klaus et al., 1991). As expression of a wheat UCP gene, *WhUCP1*, had been insensitive to low temperature in a previous report (Murayama and Handa, 2000), response of the *WhUCP1* transcript level to low temperature was unclear in our RT-PCR analysis (Fig. 2A). However, the *WhUCP1* transcript was more abundantly accumulated in M808 than in CS at most of examined time points (Fig. 2B). *WhUCP1* also seemed to contribute to cultivar difference of the cold/freezing tolerance between M808 and CS (Fig. 2B). Although the low temperature response of the *WhUCP1* transcript was not clearly observed, comparison of the

accumulation levels showed significant difference in wheat. Therefore, at least some of the mitochondrial protein genes might be associated with development of the cold/freezing tolerance. It is suggested that the extremely high level of cold/freezing tolerance of M808 is produced by sum of plural, independent pathways.

Stress induction levels of AOX have showed a considerable variation among species and approaches (McDonald et al., 2002). Low temperature induced AOX in mung bean but not in soybean (Gonzalez-Meler et al., 1999). Treatment of potato leaves with antimycin A resulted in only small increases in AOX protein accumulation and no increases in the alternative respiratory capacity, compared to the large increases in tobacco cells (Vanlerberghe and McIntosh, 1996; Geisler et al., 2004). In maize, activity of the alternative pathway was significantly increased in the more stressed chilling-sensitive cultivar than the tolerant cultivar, indicating that the alternative pathway is not related with the chilling tolerance (Ribas-Carbo et al., 2000). Interestingly, our observation in M808 and CS was completely contrary to the maize cultivar difference. Response of the alternative pathway to the cold/freezing stress in maize might be different from that in wheat cultivated in the higher-latitude area. Comprehensive, comparative study should be required to clarify the difference of low temperature response between maize and wheat in future.

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Legends of figures

Figure 1. Respiration response to low temperature in the two wheat cultivars. Results are represented as mean±standard deviations (5 independent experiments) in CS and M808. (A) Changes in the total O_2 consumption under the standard (22˚C) and low temperature (4˚C) conditions. (B) Comparison of the O_2 consumption in the cytochrome and alternative respiration pathways under the standard and low temperature conditions. Two-week-old seedlings were treated with low temperature for indicated periods. (C) Ratio of the alternative pathway to the cytochrome pathway in CS and M808. Student's *t*-test was used to test for statistical significance (**P*<0.05) between CS and M808.

Figure 2. Expression profiles of *WAOX1a*, *WAOX1c* and *WhUCP1* under low temperature condition in seedling leaves of common wheat. (A) Transcript accumulation of the three nuclear genes encoding mitochondria-targeted proteins, *WAOX1a*, *WAOX1c* and *WhUCP1*. Cycle numbers of PCR are indicated at the right sides of electrophoregrams. As an internal control, a fragment from the wheat ubiquitin gene (*UBI*) was amplified. (B) Comparison of the *WAOX1a*, *WAOX1c* and *WhUCP1* expression levels between CS and M808 under low temperature condition for each period. (C) Quantification of the *WAOX1a* transcripts. The *WAOX1a* transcript levels were quantified as relative values to the level of CS under the standard condition revealed by the quantitative RT-PCR method.

Figure 3. Accumulation patterns of mitochondrial AOX proteins under low temperature condition in seedling leaves of common wheat. Two-week-old seedlings were subjected to low temperature treatment (4˚C) for indicated periods. (A) Immunoblot analysis with the AOX antibody in CS and M808. The bottom lane shows CBB-staining gel. (B) Kinetics of the AOX accumulation levels (means±standard deviations) in CS and M808. The AOX levels were quantified as relative values to the level of CS under the standard condition. Student's *t*-test was used to test for statistical significance (**P*<0.05; ***P*<0.01) between CS and M808.

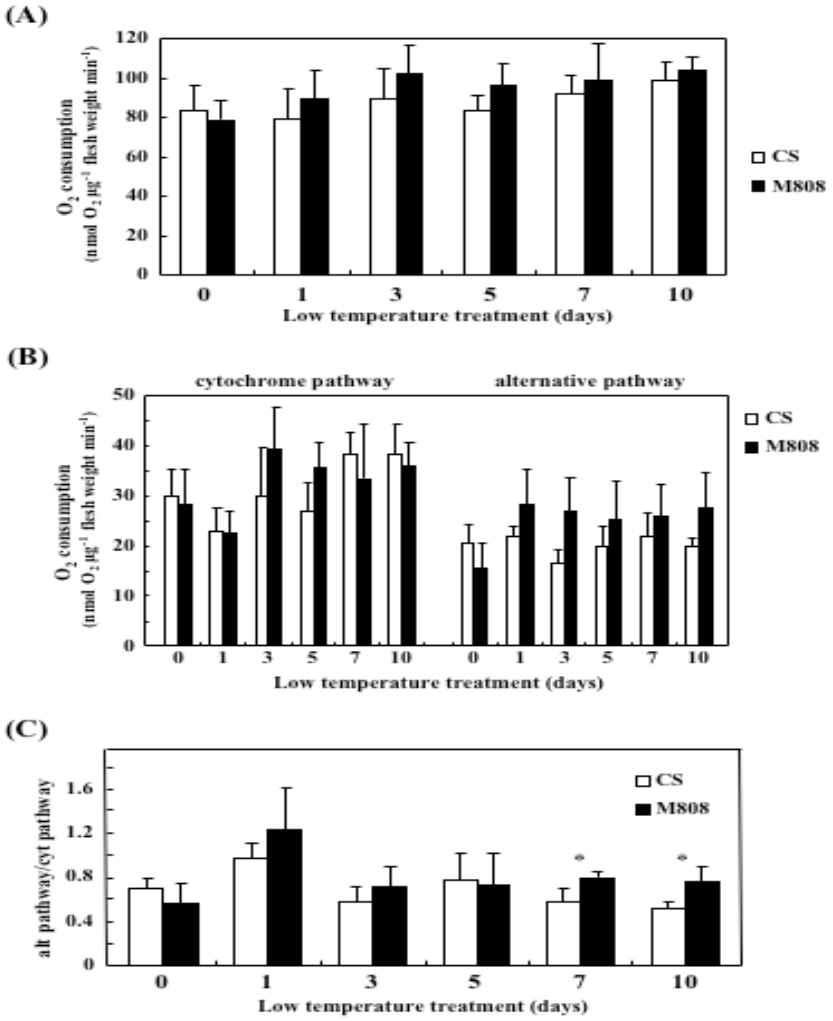
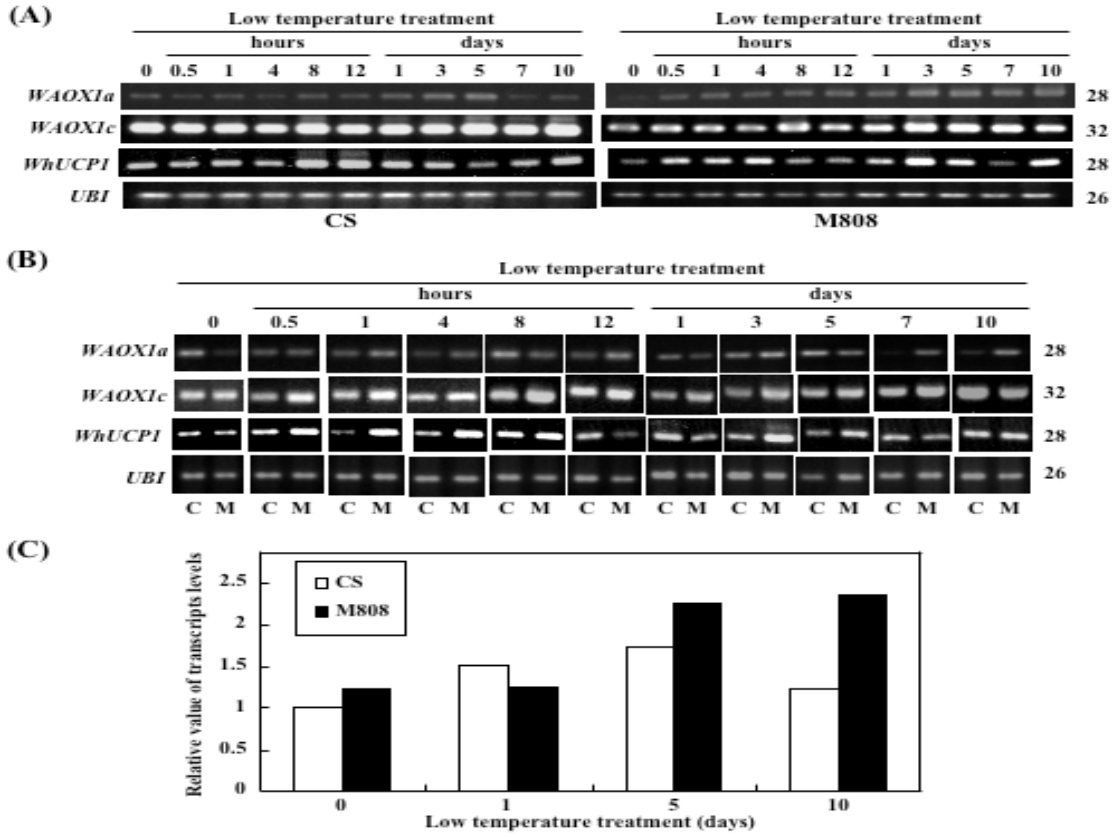


Fig. 1 (Mizuno et al.)



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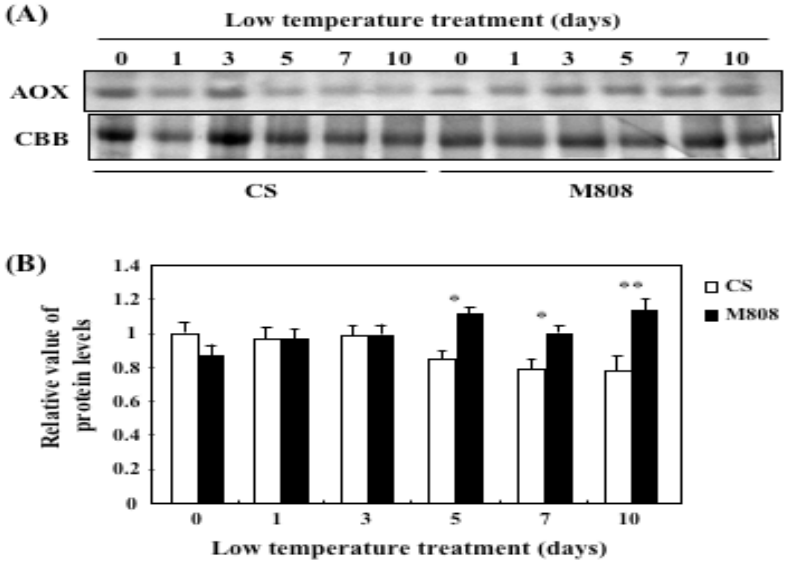

Fig. 3 (Mizuno et al.)