臺灣二ＯＯ八年國際科學展覽會

科　　　別：植物學

作 品 名 稱：Molecular and Cellular Responses under Hypoxic Stress among Rice Cultivars with Different Flooding Tolerance 缺氧逆境下不同耐淹水性稻子的分子生物及細胞反應

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作者簡介

我是莊迪喬，高雄女中二年級學生。

我生長於和樂溫暖的家庭，父母開明十分重視我們的家庭教育，是我學業和研究上最大的支柱，姊姊目前就讀於大學是我交心良友。父母每逢寒暑假即帶我們到處旅遊，或參觀美術館、博物館，以培養我們廣泛的興趣。

我喜歡參與各種活動，也熱衷科學研究，校內外科學展覽、競賽、我都熱情以赴，期許能充實自己的能力，擴大視野。音樂美術也是我的最愛，在緊繃的課業下音樂美術是我雕琢心靈最好的方法。

Molecular and Cellular Responses under Hypoxic Stress among Rice Cultivars with Different Flooding Tolerance

缺氧逆境下不同耐淹水性稻子的分子生物及細胞反應

ABSTRACT

Global warming increases the frequency of flooding, which drastically reduces the growth and survival of plants. Although rice (*Oryza sativa*) appears well-adapted to flooding of roots as it is often farmed in paddies, problems arise when the whole plant is submerged in water. I am interested in the structural and molecular responses that result in different submergence tolerances in rice cultivars. Indica rice FR13A is submergence-tolerant and frequently used in molecular breeding for this trait, while IR64 is a high-yield but submergence-intolerant cultivar. In this study, I monitored the growth rate, aerenchyma formation, and gene expressions of the carbohydrate metabolism in FR13A and IR64 seedlings subjected to submergence for 24 hours, by means of real time RT-PCR and microarray. FR13A had prominently inhibited coleoptile growth and sustained levels of aerenchyma formation whereas IR64 did not. The mRNA levels of alcohol dehydrogenase 1 (ADH1) in FR13A was induced prominently, while ADH2 was induced in IR64 during early hours of submergence. The induction of pyruvate decarboxylase 2 in FR13A was stronger than IR64. The expression of sucrose synthase was similar in both strains. Expressions of the genes involved in anaerobic carbohydrate metabolism were also studied by analyses of microarray data. My findings demonstrate that elongation quiescence, persistent aerenchyma formation and shifts in anaerobic carbohydrate metabolism gene expressions are beneficial strategies of FR13A towards submergence. Through elucidating the molecular basis of coordinating submergence tolerance genes as this study provided, it will be possible to discover multiple traits associated; hence crop improvement for flooding tolerance could be achieved.

Key words: rice, submergence tolerance, gene expression

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摘要

全球暖化造成水災頻繁，嚴重威脅植物生存。看似耐淹水的水稻，在完全淹水下亦有其生存危機。水稻 FR13A 因耐水性極佳而常用於分子育種，IR64 產量高卻不耐水。是那些特質使稻種間有不同耐水機制？我們觀察其幼苗淹水 24 小時後生長情形、通氣組織 (aerenchyma) 變化及應用即時反轉錄聚合酶鏈反應 (real time RT-PCR)，研究酒精醱酵的主要蛋白質：乙醇脫氫酶 (alcohol dehydrogenase, *ADH1*, *2)* 及丙酮酸脫羧酶 (pyruvate decarboxylase, *PDC2*) 基因之表現量。兩種水稻的胚鞘及根都因淹水減緩生長，以 FR13A 減緩最明顯。通氣組織在淹水期間都有增加，FR13A 中的形成近似於對照組，IR64 則明顯較差。FR13A 中 *ADH1* 及 *ADH2* 在淹水一小時後迅速增加 60 至 100 倍，IR64 僅增加 10 至 20 倍。*PDC2* 在 IR64 中表現量的增加幅度較大，但最大值仍小於 FR13A 之基礎表現量。由此可知，FR13A 在完全淹水時成長減緩而原有通氣組織則持續生長，且酒精醱酵中的基因有獨特誘導反應，因此耐水性較佳。藉由此研究揭開水稻細胞及分子生物學上的耐水反應策略，將可更精準地改良稻作使其對抗淹水逆境，解決未來因環境造成的糧食危機。

關鍵詞：水稻，淹水逆境，基因表現。

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一、前言

稻是世界上極古老的農作物之一，經數千年的演化，栽培、淘汰與散播，如今遍佈世界各地，不但是世界過半數人口之主食，在產米國家經濟方面更扮演相當重要之角色。一般來說，水災是造成低窪地區稻作損失的三大主因之一 (另二主因爲乾旱及土地鹽鹼化)。受水災所苦的稻作地區約佔全部稻作區的半數以上，其所造成的損失量從 10~100 %不等。全球暖化下颱風增加、豪雨頻繁，水災也日益頻繁；此外，多項數據亦顯示，世界性之糧食不足是未來不可避免的趨勢。所以提升稻米對水量的容忍度，進而提高稻米的存活率，可增加易淹水地區稻米的產量，解決食糧不足的問題。

水災 (flood) 是自然界常見且影響廣泛的災害，對大部份的陸生植物造成非常大的傷害，引起植物死亡或減少農作物收成。主要因素爲氣體在水中的擴散速率較空氣中慢了 10,000 倍，且氧氣不易溶於水。植物根部在淹水狀況下氧氣不足，代謝所產生的二氧化碳亦會累積而造成傷害。

植物的型態有可能因爲缺氧而產生變化。有些植物淹水時，莖會快速生長，伸出水面以獲得氧氣，即脫逃策略 (escape strategy)。植物莖的伸長需要能量，莖若未能及時伸出水面，反而會因消耗能量而危害到植物的生命；由低地稻米研究發現，莖的伸長與存活機會有負的相關性。因此有些植物採行減緩生長的靜止策略 (quiescence strategy)，藉以保持能量及糖份，以待水退去後再行生長 (Perata & Voesenek, 2007)。此方式較適用於極短暫淹水或長時期淹水。淹水時植物內的通氣組織 (aerenchyma) 亦會增加，以增進氣體在植物體內的運輸。通氣組織內含空氣的腔隙（Lacunae）主要有兩種型態：由細胞與細胞間分開而形成的稱爲 schizogenous aerenchyma，由細胞的塌陷而構成的稱爲 lysigenous aerenchyma；水稻的通氣組織屬於後者 (Seago JR et al., 2005)。大多數的水生植物，本質上就會產生通氣組織，且淹水時會再增加。一般認爲水稻具有這個特性 (Visser et al., 2004; Colmer, 2003)。通氣組織形成時，爲造成細胞的計畫性死亡 (programmed cell death）需要有乙烯的參與。目前已有分子生物學的証據來證明不同水稻在水下的生長及對淹水的忍受力的差別，被稱之爲乙烯反應因素

(Ethylene Response Factor) (Fukao et al,. 2006)。

若是從分子生理學上的角度來看，氧氣的缺乏會使得根細胞粒腺體的能量釋放機制，即克氏循環及磷酸化作用運作受到影響，而糖解作用成為供給 ATP 的唯一來源。糖解作用所產生的 NADH 通常由粒線體內的磷酸化作用所消耗而還原為 NAD+，但在缺氧的狀況下粒線體內的磷酸化作用無法進行；細胞為了持續唯一的 ATP 來源─糖解作用，必須找到路徑來回收 NADH 為 NAD+，而乳酸醱酵及酒精醱酵便提供了這麼一個管道。乳酸醱酵是將原先會在粒線體中被轉化為 Acetyl CoA 的丙酮酸經 lactate dehydrogenase (LDH) 轉為乳酸，並在過程中將 NADH 轉為 NAD+。酒精醱酵則是將丙酮酸經由 PDC 轉為 Acetaldehyde，而後經由 ADH 轉為酒精，並在轉為酒精的過程中將將 NADH 轉為 NAD+。在這種狀況下所產生的 ATP 因為僅有糖解作用而無粒線體內的作用，所得的 ATP 僅為平常的十八分之一。LDH 適合在一般細胞內的 PH 值中作用，而 PDC 適合於較一般細胞內 PH 值低時作用，所以細胞會先啓動乳酸醱酵；由於乳酸醱酵會使得細胞內的 pH 值降低，其後將迅速被抑制而轉為酒精發酵 (Taiz & Zeiger, 2006) (Figure 1)。

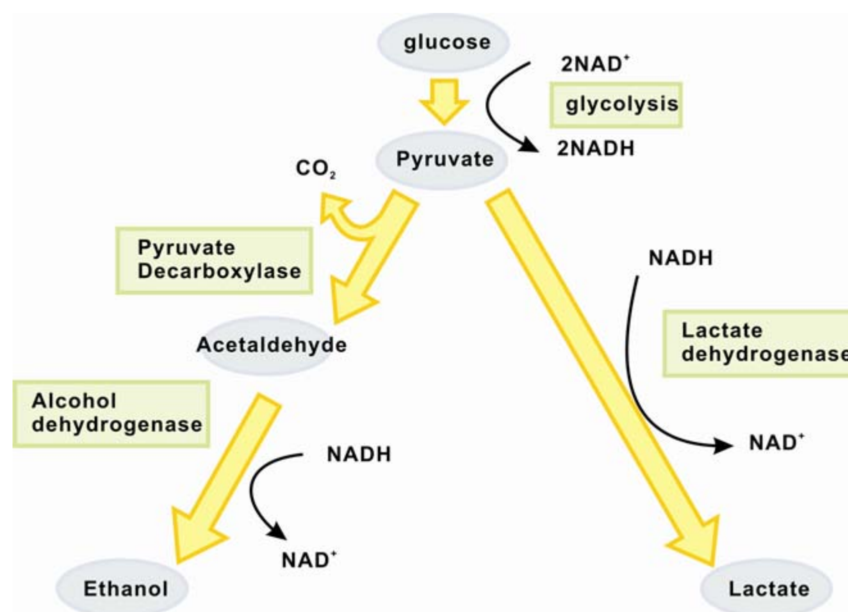

Figure 1. Major products of carbohydrate metabolism in flooded roots.

Pyruvate produced by glycolysis is initially fermented to lactate during the early phase of oxygen distress. Proton production by glycolysis will decrease the cytosolic pH. At lower pH, lactate dehydrogenase (LDL) activity is inhibited, and pyruvate decarboxylase (PDC) is activated. As a consequence of lower pH, the fermentation to ethanol increase and to lactate decrease. The pathway of ethanol fermentation uses up more protons than does the pathway of lactate fermentation. The cytosolic pH will gradually increase and this will enhanced the ability of the plant to cope with the episode of anoxia. (Figure drawn by author)

1980 年代聚合酶鏈反應 (Polymerase chain reaction, PCR) 應用於 DNA, RNA 及蛋白質層面的表現分析使得分子生物學蓬勃發展。反轉錄酶聚合酶鏈反應 (Reverse Transcription-Polymerase Chain Reaction, RT-PCR) 則是指使用反轉錄酶 (reverse transcriptase)，把 RNA 先轉錄成 DNA，再進行聚合練酶反應；由 RNA 轉換爲 DNA 稱爲反向轉錄。由此便可以由傳統的 PCR 作業得到細胞內 RNA 的表現情形。近年更將 PCR 技術與螢光的偵測結合在一起，稱爲即時聚合酶鏈反應 (real-time PCR)。Real time RT-PCR 利用非常敏感的螢光染劑 (例如 Sybr Green) 與試管內形成的雙股 DNA 結合並且產生螢光，由電腦記錄每一個循環釋出的螢光指數。由於聚合酶鏈反應會造成 DNA 成對數性的增加，螢光指數也會隨著 DNA 的複製而增加，螢光指數突然開始快速增長的某個循環 (這個加速的起點是由一個叫做 threshold 的值來定義)，稱爲 Ct (cycle of threshold)，與起初 cDNA 的含量成反比，因此能推測當初的 RNA 含量，進而較傳統 PCR 更精確的研究細胞內基因表現的變化 (Gachon et al., 2004)。

水稻在對抗逆境時會有結構上及代謝上的改變，如通氣組織的形成、莖節間的增長以促進氣體的擴散及流通、及進行不需氧氣的醱酵作用，以持續產生 ATP。目前基因表現的研究上已能發現某些基因或蛋白質，在逆境下可能的表現，但精確機制仍未知。因此本研究運用 real time RT-PCR，研究對淹水有不同耐性的兩種水稻，於種子的最初萌芽生長階段，在低氧環境下發生糖解作用改變時，所牽涉到的基因表現，及低氧環境下通氣組織的形成是否有受到不同的影響。希望在結構上或分子生物學上能更進一步的了解，發現它們之間不同的特性，不但可以釐清分布在世界上不同區域的不同植物，是以何種不同的適應機制去適應不同的環境與逆境抗性，且可以運用在改善稻米對抗逆境，希望能提供解決未來不可避免的世界性糧食不足問題。

二、研究方法或過程

(一) 實驗稻種及育苗的準備

1. 實驗稻種

在植物分類學上，稻屬於種子植物門 (Phylum *Spermatophyta*)，被子植物綱 (Class *Angiospermae*)，單子葉亞綱 (Subclass *Monocotyledoneae*)，穎花目 (Order *Glumiflorae*)，禾本科 (Family *Gramineae* or *Poaceae*)，稻屬 (Genus *Oryza*)，共有 22 個種 (species)。其中亞洲型稻種 *Oryza sativa. L.*由於其產量高，廣泛分布於世界主要稻作產區，又可分爲三個亞種：(1) 印度型稻 (indica)：又稱爲秈稻 (Hsien)，通常分布於熱帶地區。(2) 日本型稻 (japonica)：又稱爲粳稻 (Keng)，能抗較低溫的環境，通常分布於溫帶地區。(3) 爪哇型稻 (javaonica)：又稱爲熱帶型粳稻 (tropical japonica)，栽培面積最少 (郭 & 范，2007)。

Table 1. Rice genotypes used in the present study

Cultivar	Ecosystem	Submergence phenotype	Subspecies	Origin
FR13A	Lowland	Tolerance	indica	IRRI
IR64	Lowland	Intolerance	indica	IRRI

IRRI, International Rice Research Institute.

本實驗使用的兩種水稻FR13A及IR64皆屬 *Oryza sativa. L.*。FR13A爲印度古老的耐水淹稻子，IR64 爲目前東南亞廣爲種植高產量的水稻。以上兩種稻種的種子由中研院植微所提供，其取自 IRRI。

要育苗時，將種子去除外殼並以 1 %的 NaOCl 消毒 3 分鐘，再用清水加以反覆洗淨。先在乾淨的 3M 濾紙上用美工刀切出條狀切痕，而後將消毒過的水稻種子懸掛在兩層浸濕濾紙上面。濾紙附在透明壓克力板上，而板子垂直固定於有蓋的塑膠盒內，而後將塑膠盒放入 30℃恆溫的植物培養箱，保持在黑暗下，內置杯水維持適當濕度 (Figure 2)。

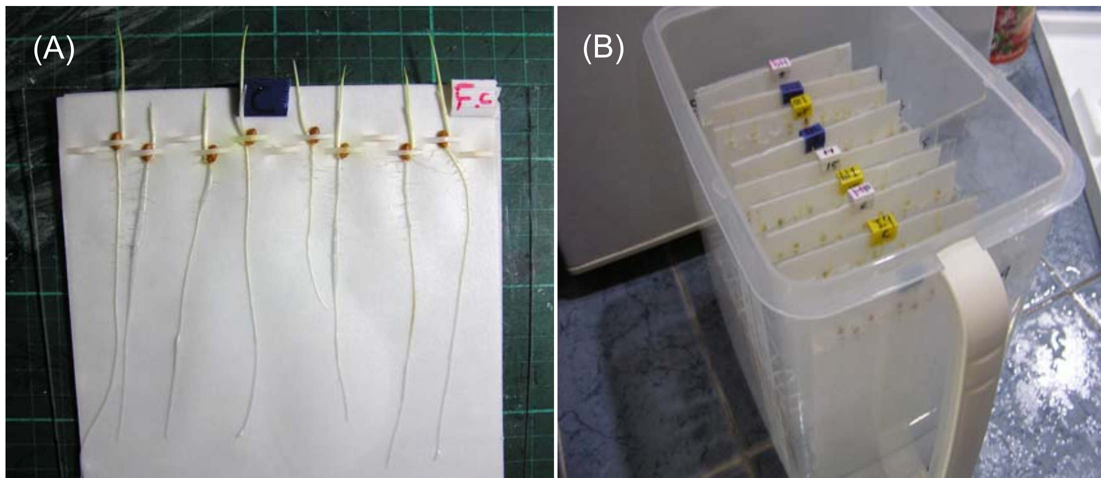

Figure 2. Experimental box, acrylic board and the 3M filter paper.

(A) Seeds germinated while attached to the wet 3M paper and the acrylic boards were placed vertically over plastic box (B). The water below was just touching the lower end of filter paper. The box lid is not shown.

（二）淹水後的生長情形 (Desubmergence experiment)

將 3 天大幼苗分成二組，其中一組完全淹水 24 小時後，將水退去，持續培養到第 8 天，記錄完全淹水前後胚鞘的長度變化，並分析二組生長情況的差別。

（三）淹水實驗 (Submergence experiment) 及通氣組織的探討

3 天大的幼苗分爲二組，淹水處理組及對照組。淹水處理組爲整個塑膠盒裝滿水，全株完全浸入水中 24 小時，記錄完全淹水前後初生根及胚鞘的長度變化。並在 24 小時後收集兩組初生根放入 FAA solution 保存，並徒手製作初生根切片觀察其通氣組織的形成。FAA solution 的調配比率爲，福馬林 (formalin)：冰醋酸 (glacial acetic acid)：50 %~70 % 酒精 = 5：5：90 (v/v/v)。

將徒手切片的根的横切面放在 Leica 光學顯微鏡下觀察，並以 Olympus 數位相機拍下照片。其後以 Adobe Photoshop CS2 計算通氣組織所佔面積的像素 (pixels) 佔整個皮層的百分比作爲比較的根據。

(四) *ADH* 及 *PDC* mRNA

將 3 天大的幼苗分爲二組，實驗組爲完全浸入水中，而在第 0、12 及 24 小時收集根部，即刻以液態氮急速冷凍後，保存於－80℃的冰箱中，以 Real time RT-PCR 分析 *ADH1*、*2* 及 *PDC2* mRNA 的反應狀況。

1. RNA extraction

將稻子的根在液態氮中磨成粉，倒入裝有 1ml Trizol 的 1.5ml ependorff 中，置於室溫 5 分鐘後加入 200μl chloroform 並劇烈搖晃 (vortex)，再置於室溫 5 分鐘。在 4℃以 12000G 離心十五分鐘後取上清液約 400μl 置入新的 eppendorff，加入 100μl isopropanol 及 30μl high salt solution (1.2M Sodium Citrate, 0.8 M NaCl)，輕輕搖晃混勻，置於室溫 10 分鐘。在 4℃下以 12000G 離心，沉澱出 RNA pellet。除去液體，並加入 1ml 75 %酒精，劇烈搖晃 (vortex)，再離心。以 100 %酒精重複上列動作。除去液體並風乾 RNApellet 後將 RNA 回溶於 DEPC 水，即可測量 RNA 濃度及品質以利後續反應。

2. RNA 反轉錄

萃取出的 RNA 在反轉錄之前先以 DNase 處裡過；即將 DNase (Ambion Super DNase) 及 10Xbuffer 置入 37℃恆溫槽內 20 分鐘，再加入 inactivation reagent。將全部的 RNA 混合 oligo (dT) 及 dNTPmix (Roch) 後加 DEPC 水到 12μl，加熱到 65℃五分鐘後插入碎冰冷卻。其後再加入 5X first strand buffer (Invitrogen)、DTT (USB)、RNase OUT (Invitrogen) 及 Reverse Transcriptase (Invitrogen MMLV)，置於 37℃50 分鐘，接著至於 70℃15 分鐘。

3. Real time polymerase chain reaction

5mg 的 cDNA 混合 sybrgreen mastermix (ABI) 及 primers 並以 DEPC 水稀釋後加入 well 中。本實驗使用 ABI Prism 7000，並設定如下：

Stage 1：50℃ hold, 2 minutes → 95℃ hold, 10 minutes

Stage 2：95℃, 15 seconds → 60℃, 60 seconds 重複 35 次

Stage 3：降溫至 4℃。

4. Primers

Gene	AG Number	Forward primer	Reverse primer
ADH1	Os11g10480	ATTGACCTGAACGCCAACAG	CCAGGATACACAGAAGAACCGTA
ADH2	Os11g10510	GCCGACCAAGGATGATGT	CCCTGTCTCACGACCATTAT
PDC2	Os03g18220	GCTGGGCTCAATCATAATAA	TGGAGAAACAGATGTTCACA

5. 本文使用 ABI Prism 7000 SDS 及 $2^{-\Delta\Delta Ct}$ method 進行 RNA 相對表現量的計算進行分析與比較 (Figure 3)。

(1). 在所輸出的結果的 amplification plot 中將Threshold取至一段所有曲線看起來平直且互相平行的位置進行進行分析，取得Ct值。

(2). ΔCt:

ΔCt = Ct (candidate gene) - Ct (internal control gene)

(3). ΔΔCt:

ΔΔCt = ΔCt (Treatment X) - ΔCt (Treatment 1)

(4). RNA 表現的相對量 = $2^{-\Delta\Delta Ct}$

當 $2^{-\Delta\Delta Ct} < 1$ 時即爲down regulate，當 $2^{-\Delta\Delta Ct} > 1$ 時即爲up regulate。

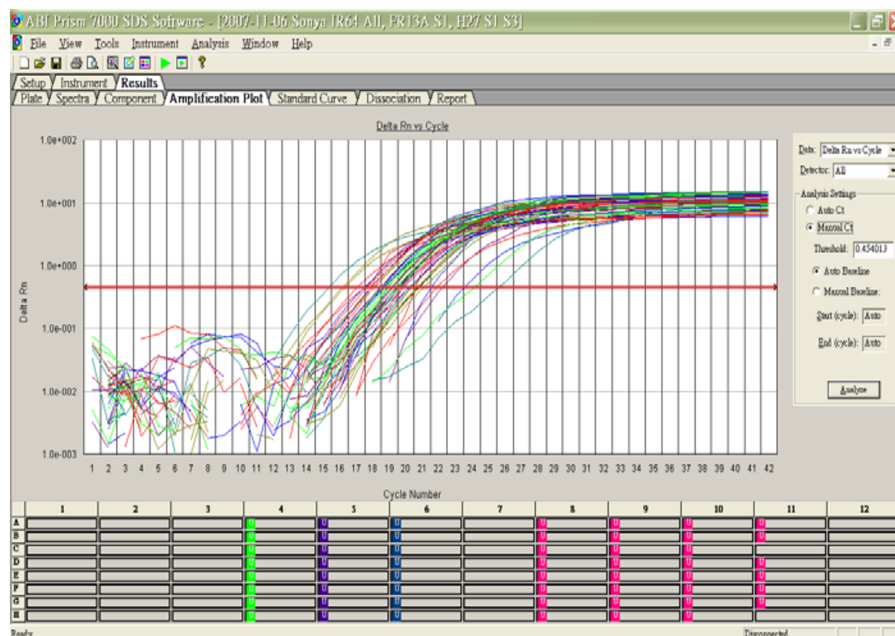

Figure 3. Real-time RT-PCR analysis

The $2^{-\Delta\Delta Ct}$ method is used to calculate the relative expression of genes. A decent threshold (the horizontal red bar across the curves) is chosen for data analysis. ΔCt is calculated by Ct (candidate gene) - Ct (internal control gene), ΔΔCt calculated by ΔCt (Treatment X) - ΔCt (Treatment 1). The relative amount of target is presented as $2^{-\Delta\Delta Ct}$.

三、研究結果及討論

(一) The effect of 24 hours submergence on the seedling

Figure 4. FR13A rice seedlings approximately 3 days after germination.

Seedlings grew in continuous darkness while positioned on wet filter paper in aerated condition. Primary root and the coleoptile were clearly demonstrated. Primary root length was measured from the root-embryo junction to the root tip. Coleoptile length was measured from the middle of the coleoptile node to the tip of the coleoptile. White bar = 1 cm.

1. Coleoptile elongation

根 (primary root) 是最早從種子冒出的組織，接著可見胚鞘 (coleoptile) 的出現 (Figure 4)。胚鞘的長度是測量其尖端至種子冒芽處，測量的時間點爲 3 天大幼苗在淹水前，及三天大的幼苗在完全淹水 24 小時後。

雖然二種水稻的胚鞘皆有生長，但其生長量均較對照組低 (Figure 5A)。FR13A 第四天所增加的長度爲 4.6 ± 0.4 (mean ± SE)，佔對照組的 28.8 %，而 IR64 增加長度爲 10.4 ± 0.6 (mean ± SE)，佔對照組的 49.3 %。FR13A 在完全淹水的環境下，胚鞘的生長變化較 IR64 還要大。

2. Primary root growth

根長定義爲根尖至根與種子的交接處的長，其測量的時間點爲 3 天大的幼苗及其完全淹水 24 小時後。雖然兩種水稻的根在淹水的狀態下皆有生長，但生長量均較對照組低 (Figure 5B)。FR13A 第四天所增加的長度爲 10.5 ± 0.6 (mean ± SE)，只有對照組在同樣 24hrs 中所生長的 22.8 %，而 IR64 長度爲 12.4 ± 0.9 (mean ± SE)，爲對照組所生長的 37.4 %。

FR13A 在缺氧環境下，根的生長明顯的受到較大的限制；因此可以說耐淹水稻種在淹水的狀況下根的生長所採取的策略與胚鞘是相同的。所以 FR13A 在低氧環境所採取減緩胚鞘及根的生長，達到保存能源，減少碳水化合物的消耗，是非常重要的。這樣才能增加淹水時的抵抗能力 (Setter & Laureles, 1996)。

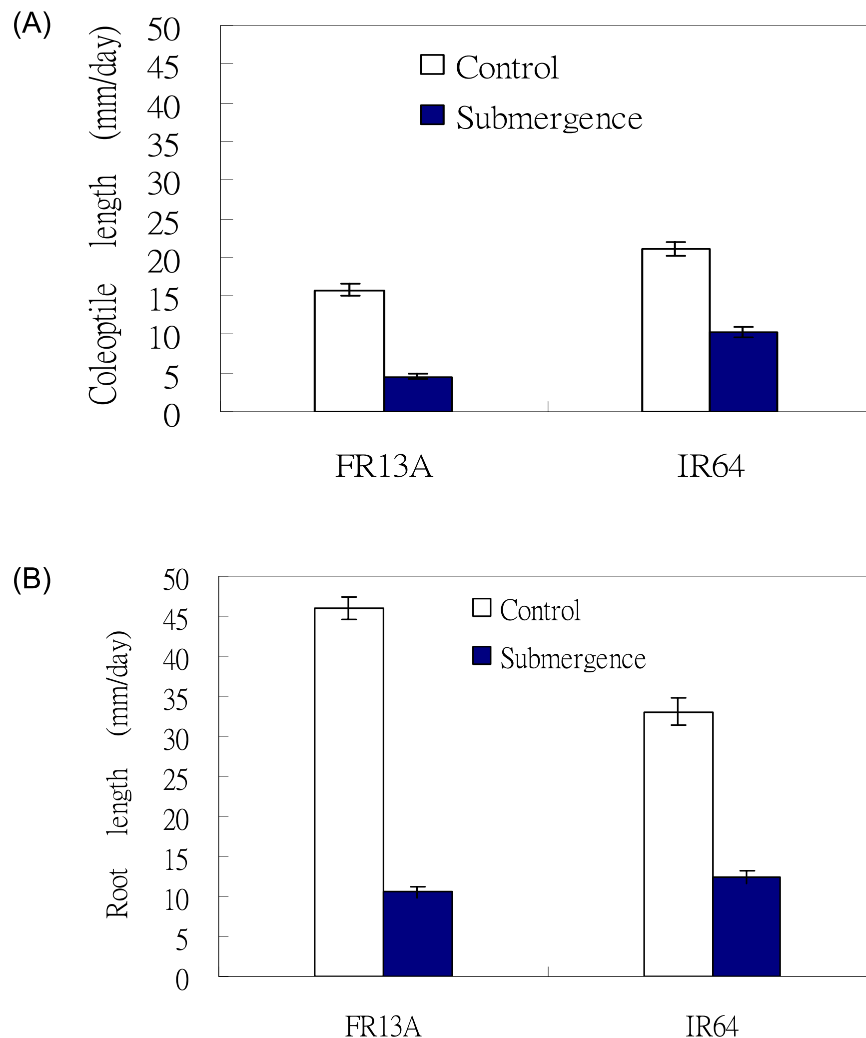

Figure 5. Effects of one day hypoxia on coleoptile and root growth of 2 rice cultivars.

Seedlings were germinated on two sheets of moist filter paper in darkness at 30°C in a growth chamber. Three-day-old rice seedlings were then completely submerged for 24 hours. The control group was left in the original condition for the same length of time. The length of coleoptiles (A) and primary roots (B) were measured with a ruler before and after the 24 hours hypoxic treatment. Elongation during the treatment was determined by subtracting the length before treatment from the length after treatment. Values are mean ± SE of the coleoptile of 20 seedlings.

(二) 淹水 24 小時之後的繼續生長觀察

(The recovery seedling growth after 24 hours submergence)

本實驗是將三天大的幼苗淹入水中 24 小時，之後再將幼苗移回原本生長的環境中繼續培養至幼苗九天大 (即三天的正常生長、一天淹水處裡、五天正常生長)。

當淹水 24 小時的幼苗脫離淹水後，FR13A 及 IR64 均有恢復原來的生長趨勢 (Figure 6)。第 8 天後胚乳消耗殆盡，胚葉生長趨緩。IR64 雖爲不耐水淹品種，但卻有較佳的生長，可能原因爲 IR64 本即爲一株生命旺盛生長能力強的培育種，且短暫的淹水時間並不足以產生決定性的影響。若仔細去注意生長後期 (第七天以後的生長)，可以注意到受過淹水處理的 IR64 的生長情形明顯趨緩，而受過淹水處理的 FR13A 並沒有顯示此疲態；因爲本實驗並沒有照光亦沒有補充養分，由此可以推測 IR64 在淹水時所消耗的胚乳確實較多。

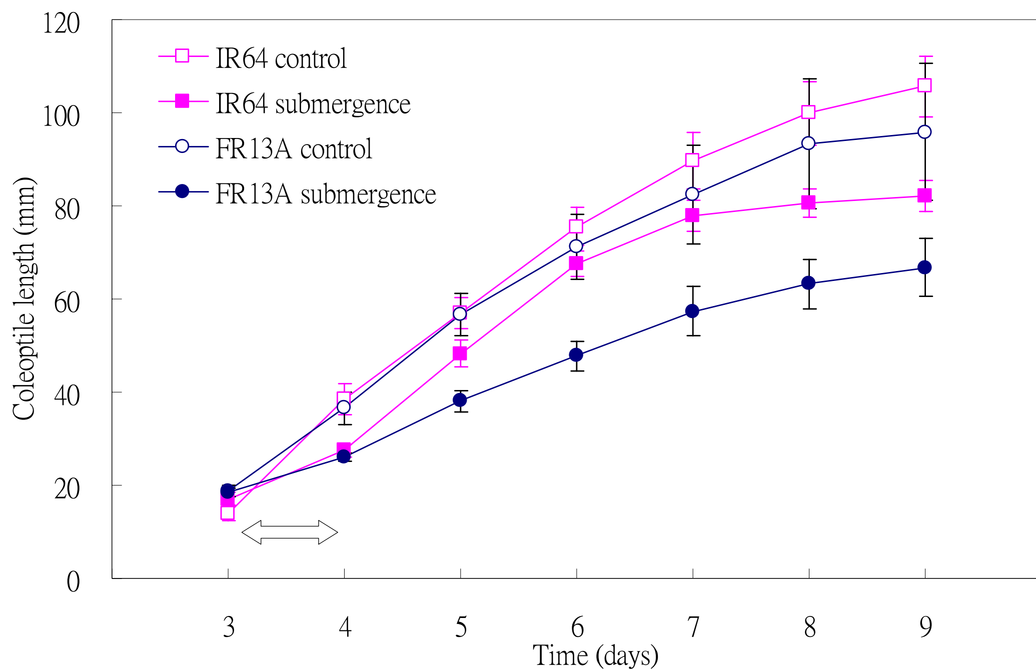

Figure 6. Effect of one day hypoxia on coleoptile growth of rice seedlings.

Three-day-old seedlings were completely submerged for 24 hours and then allowed to recover for another 5 days. The coleoptile lengths were recorded daily. The primary root was kept from reaching the water below after desubmergence. Values are mean lengths (± SE) of the coleoptile of 10 seedlings. The two headed arrow indicates the period of the 24 hours hypoxic treatment.

(三) Root Anatomy

在這兩個品種間，根的構造及發育並無特別差異，因此以 IR64 的根的橫切片來代表。種子的初生根 (primary root) 可以分為根帽、生長部、延長部及成熟部。在成熟部可以看見明顯的根毛從表皮長出。根內部的皮層中隨處可見散佈在薄壁細胞間的柱狀空間，即是通氣組織 (aerenchyma)。一般來說，通氣組織要在根的後方約十公分才漸漸成熟 (Kawai et al,.1998)；若將根分成三等份來進行切片觀察，便可更明顯的看到此種變化。愈接近根尖的通氣組織量愈少，而愈近種子處愈多 (Figure 7)。根外皮的構造由外至內如下 (Figure 8A)：rhizodermis，ectodermis，sclerenchyma 及一層 cortical cell layer，根的中心為 stele (Figure 8B) (Ranathunge et al., 2003)。

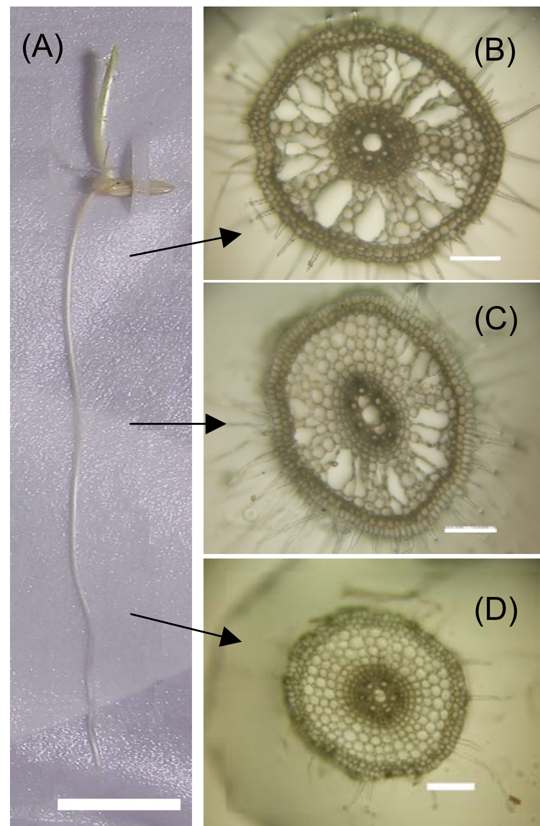

Figure 7. Free hand sections showing that the formation ratio of aerenchyma decreases along the way as it is closer to the root tip.

(A) Free hand sections were made in three parts of the primary root, as shown on above. Bar = 2cm.
(B) The nearer to the seed, the larger the aerenchyma ratio (the huge gaps). Spoke-like-structures which consist of remaining parenchyma cells or cell walls connect the stele to the epidermis. Bar = 0.1 mm.
(C)The aerenchyma ratio gradually decreases as free hand sections were made nearer to the tip (D). Bar = 0.1 mm.

(四) Aerenchyma change after episode of submergence

淹水下的植物，在新的或是舊的根內部，通常都會有縱向的細胞空隙，稱為通氣組織，通氣組織可在根、匍匐根、莖或是葉柄中都可出現。通氣組織可分為二種。schizogenous aerenchyma：藉由分開皮層或是周皮間的細胞，來增加空隙。lysogenous aerenchyma：藉由溶解部分的細胞而形成的通氣組織 (Seago JR et al., 2005)。

各種植物的通氣組織不盡相同，有其特定的分類方式。 一般來說，通氣組織被認為可幫助植物莖的生長，使植物在低氧的情況下生存，因其可以降低氧等其他氣體運送到植物各處的阻力，也可以增加氧氣的儲存空間。許多生長在僅有根部淹水的植物，會形成較大的通氣組織。一些不具耐水性的植物，如玉米、小麥、大麥等，生長在容易暴露在空氣中的土壤，通氣組織並不大；但若是生長再低氧的情況下時，通氣組織很快的就會形成。在許多的植物中由低氧所引發 ethylene 的產生被認為是要幫助通氣組織的產生。

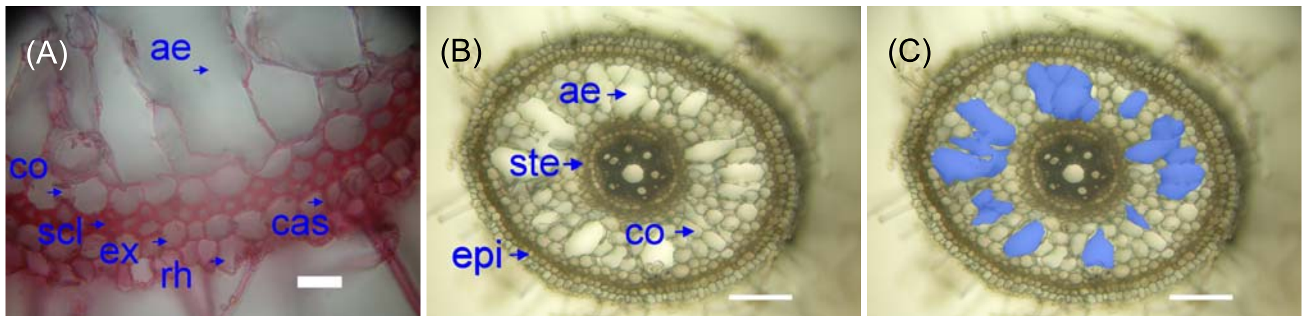

Figure 8. Detailed structure of the root and determination of aerenchyma gas space (% of the cortex).

(A) The outer part of roots contained four cell layers: rhizodermis (rh), exodermis (ex), sclerenchyma (scl) and one cortical cell layer. Freehand cross-section stained with safranin O. ae = aerenchyma. Bar =0.02 mm.
(B) A transverse root section that will be used for aerenchyma formation calculation. Shown in this picture: the epidermis (epi), the cortex (co), the stele (ste) and the aerenchyma (ae). Aerenchyma spaces are included within the cortex, as it is formed by lysis of parenchyma cells within this layer.
(C) Each area determined as the aerenchyma is brushed with a blue hue with using Adobe Photoshop CS2. The number of pixels within each filled area is calculated. The percentage of cortex area occupied by gas space is determined and is regarded as the ratio of aerenchyma. Bar = 0.1 mm.

1. The calculation of aerenchyma

將徒手切片的根的橫切面放在 Leica 光學顯微鏡下觀察，並以 Olympus 數位相機拍下照片。其後以 Adobe Photoshop CS2 計算通氣組織所佔面積的像素 (pixels) 佔整個皮層的百分比，即氣室/皮質範圍百分比，作爲比較通氣組織量的根據 (Figure 8C)。

2. Effect of 24 hours submergence to developed aerenchyma.
(水淹對於原有通氣組織的影響)

若將幼苗初生根淹水前後的變化加以分析 (Figure 9)，可以發現 FR13A 通氣組織變爲 34.63 ± 3.82 % (mean ± SE)，爲對照組的 88.48 %，而 IR64 爲 47.46 ± 2.60 % (mean ± SE)，爲對照組的 72.20 % (Figure 10)。

Aerenchyma change

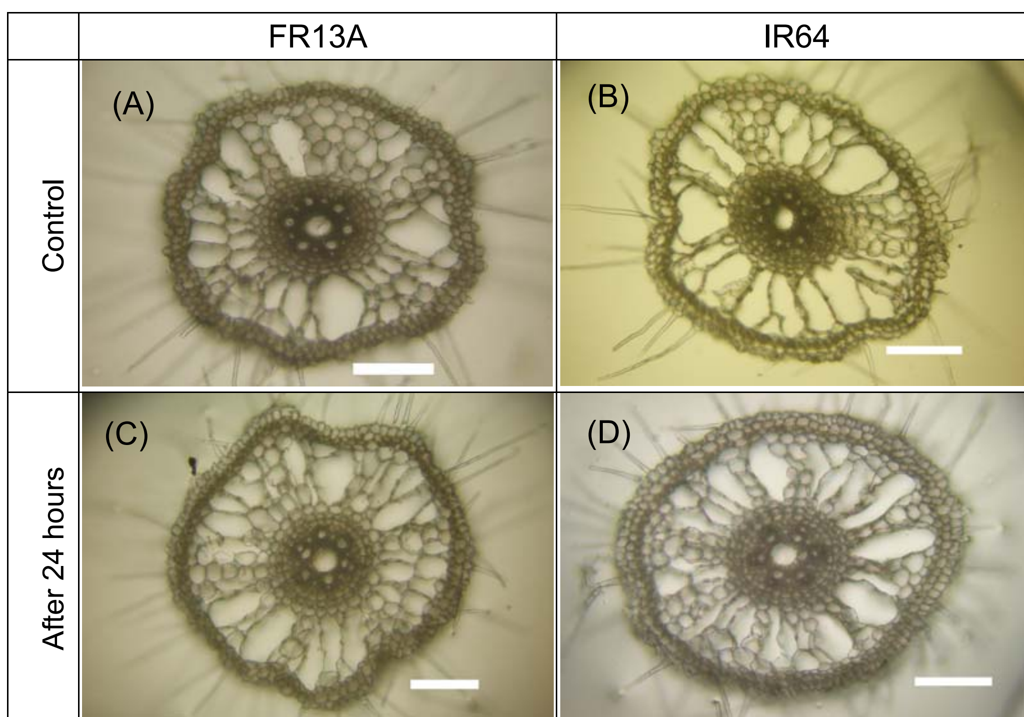

Figure 9. The increase of aerenchyma after 24 hrs submergence.

Representative transverse root sections from four roots showing formation of aerenchyma in cortex. Shown are root sections from four day old seedlings of FR13A and IR64. The control group (A), (B) were in the aerated condition and the submerged group (C), (D) underwent submergence treatment for 24hrs at three days' age). FR13A formed a similar amount of aerenchyma compared with the control group under submerged conditions while IR64 showed less amount formation. Bar = 0.1 mm.

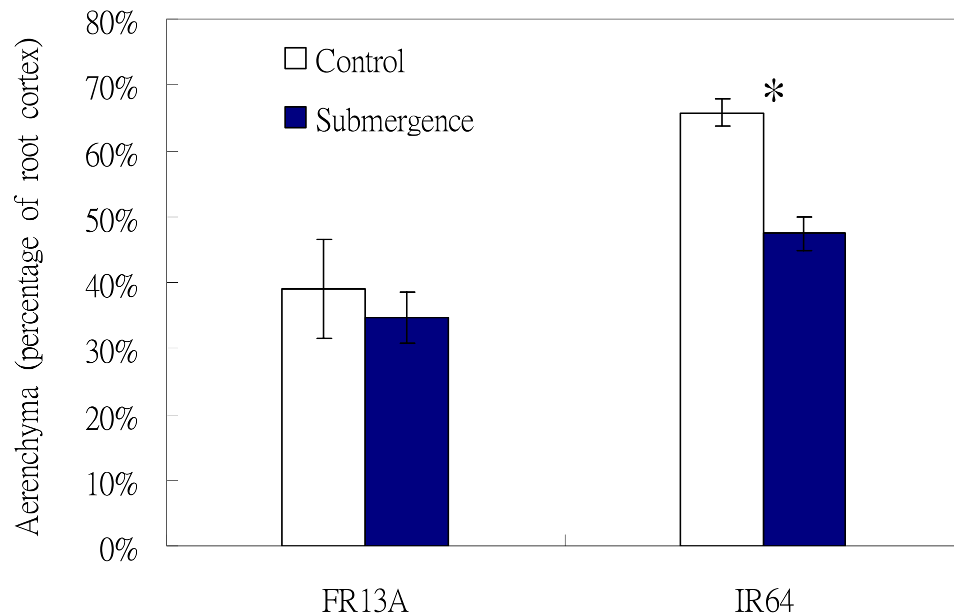

Figure 10. The change of aerenchyma after 24 hours submergence.

The sectioning site was determined by subtracting the elongation length of the last two days from the tip of the root. The aerenchyma formation percentage was calculated by the method mentioned in Figure 8. It may be taken into consideration that the aerenchyma percentage within the FR13A is less compared with IR64 because IR64 had elongated more in the day of submergence and that aerenchyma is the closer to maturity the farther it is from the root tip. The asterisk indicates that the IR64 seedling which had endured 24 hours of submergence had significantly less percentage of aerenchyma formation as compared with the control group ($P < 0.001$).

FR13A 通氣組織持續的增加，但 IR64 通氣組織的形成則受到較大的壓抑。因此可以推論 FR13A 在淹水後初生根的生長度雖較少，但仍持續增加其舊有通氣組織；IR64 則無此現象。有研究指出水稻初生根的通氣組織約在根尖後 8 至 10 mm 處開始形成 (kawai et al., 1998)。這可以解釋爲何 FR13A 的對照組中，通氣組織的比例略遜於 IR64。IR64 的生長原本就比 FR13A 還要快，若以「絕對根齡」(即推算根各部份組織的年齡，以確認各樣品所取得橫切面爲同年齡的組織) 來取得橫切面 (如本實驗)，在 FR13A 中所取的橫切面會較在 IR64 中所取得的橫切面還要接近根尖；即其通氣組織的成熟度較低。值得注意的，儘管如此，淹水狀況下 FR13A 所形成的通氣組織仍較 IR64 還要更接近自身對照組所生成通氣組織的比例。若再將 FR13A 在水中停滯生長，IR64 在水中仍持續生長 (即在淹水狀況下 FR13A 中橫切面的位置又較 IR64 接近根尖許多) 列入考量，而把 FR13A 形成通氣組織的能力與 IR64 相比，其優劣不言而論。

目前對於初生根通氣組織的研究大多以莖在水面上，而根在水面下或

土壤內淹水的情況進行實驗，結果均顯示根在缺氧環境下，通氣組織均有增加 (Colmer, 2003)；而我們的實驗爲整株幼苗均沉入水中，此時新生組織的通氣組織均減少。對於淹水前已形成的通氣組織，FR13A 有如對照組一樣的增加，而 IR64 則明顯的受到影響。許多稻子及水生植物對於淹水的反應是快速伸長莖，直至冒出水面以獲得氧氣，而經由植物體內的通氣組織結構擴散到其他部位。在某些些植物中，例如稻子，通氣組織可以是與生俱來的，當種子發芽成長時，即會形成。在玉米及其他植物，通氣組織只有在特別的環境才會被誘導出來 (Liao & Lin, 2001)。

(五) PDC and ADH mRNA expression during submergence

細胞在缺氧環境下，必須要進行醱酵作用以回收NAD+，才能繼續維持糖解作用。乳酸醱酵是將原先會在粒線體中被轉化爲Acetyl CoA的丙酮酸經LDH轉爲乳酸，並在過程中將NADH轉爲NAD+。酒精醱酵則是將丙酮酸經由PDC轉爲Acetaldehyde，而後經由ADH轉爲酒精，並在轉爲酒精的過程中將NADH轉爲NAD+。在這種狀況下所產生的ATP因爲僅有糖解作用而無粒線體內的作用，所得的ATP僅爲平常的十八分之一。LDH適合在一般細胞內的PH值中作用，而PDC適合於較一般細胞內PH值低時作用，所以細胞會先啓動乳酸醱酵；由於乳酸醱酵會使得細胞內的pH值降低，其後將迅速被抑制而轉爲酒精發酵。酒精醱酵的增加與丙酮酸脫羧酶 (pyruvate decarboxylase, PDC) 及乙醇脫氫酶 (alcohol dehydrogenase, ADH) 相對應的基因表現量有密切的關係。

本實驗使用即時反轉錄酶聚合酶鏈反應來進行基因表現亮的測量。即時反轉錄酶聚合酶鏈反應 Real-time RT-PCR (Reverse transcription - Polymerase chain reaction) 爲一已廣泛應用於分子生物領域之新興技術，可省去傳統RT-PCR 之後所必須的瓊膠電泳分析。可以節省等待結果的時間，還可降低因跑膠時DNA污染導致誤判的可能性，及避免因爲跑太多PCR cycle 導致所得的最後結果受一開始所加入dNTP量的影響大於真正RNA的量。

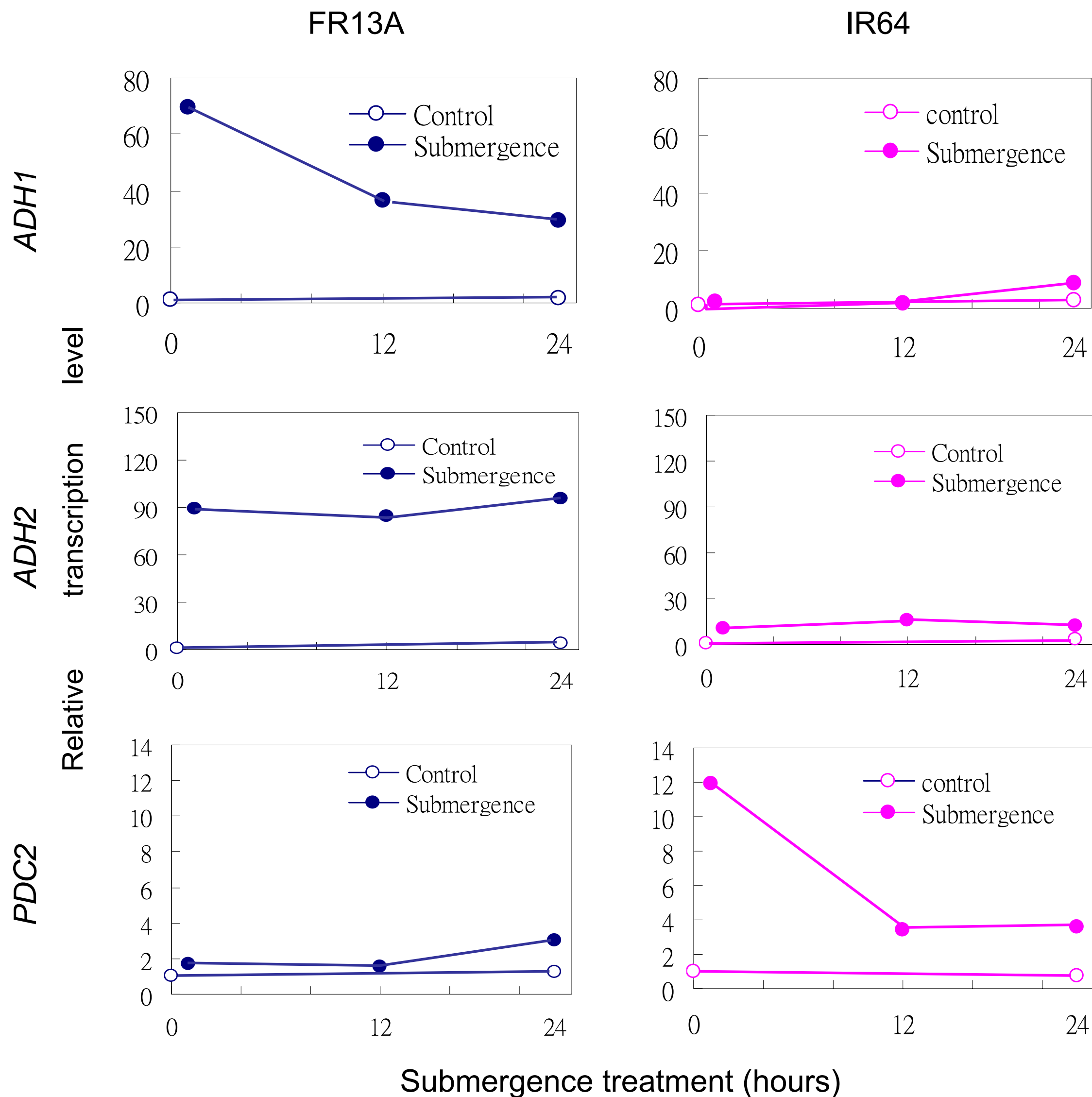

Figure 11. Effects of submergence on the expression of *ADH1*, *ADH2* and *PDC2*.

Three-day-old seedlings were completely submerged for 1, 12 and 24 hours then root samples were collected to examine the expression pattern of *ADH1*, *ADH2* and *PDC2*. Relative transcription level is shown (1 = expression data of three day old seedlings from the control group). It should be noted that expression levels of the control groups of IR64 and FR13A are significantly different. IR64 has almost twice the basal expression of *ADH1* compared with FR13A, and 4 folds of *ADH2* basal expression in comparison with FR13A; FR13A has approximately five folds of *PDC2* basal expression compared with IR64. IR64 and FR13A have different expression patterns of *ADH1*, *ADH2* and *PDC2*. The response of *ADH1* transcription in IR64 is up-regulated slightly at 24hrs. Expression in FR13A peaks at the first hour of submergence. *ADH2* transcription within IR64 due to submergence is relatively mild with only a ten fold up-regulation against submergence, compared with a nearly 100 fold up-regulation within FR13A. The up-regulation is constant, with no significant peak nor slope within both cultivars, regardless of the absolute amount of mRNA. IR64 had a small peak of *PDC2* up-regulation after the first hour of submergence, with the absolute amount of that of FR13A's baseline, then the expression sloped down. *PDC2* within FR13A was continuously up-regulated throughout the 24hrs of submergence.

本文對於 FR13A 及 IR64 的三天大幼苗在完全淹水處裡後的第一、十二及二十四小時進行 real time RT-PCR 分析，了解 IR64 與 FR13A 中 *ADH1*、*ADH2* 與 *PDC2* 對淹水時的最早期表現的不同之處。結果如上 (Figure 11)；淹水時 FR13A 與 IR64 中 *ADH1*、*ADH2* 與 *PDC2* 皆有誘導增加表現量，但其所表現的趨勢不盡相同。FR13A 中 *ADH1* 的表現量在淹水後第一個小時就達到最高峰，其後便漸漸下降，而 IR64 中 ADH1 的表現量要在淹水處理後第二十四小時才有提升。值得注意的是，IR64 中 *ADH1* 的基礎表現量在對照組 (未受淹水處理) 中的表現量爲 FR13A 對照組的兩倍。儘管如此，當 FR13A 受到淹水處理時其 *ADH1* 表現量皆較 IR64 高。

相較於 FR13A 中 *ADH2* 表現量增加爲基礎表現量的近百倍，IR64 僅較基礎表現量多出約莫十倍的表現量。*ADH2* 在淹水的組別中不論是 FR13A 或是 IR64 中，表現量都沒有很明顯的最高時間點。

FR13A 中 *PDC2* 的表現量要在淹水處理後第二十四小時才有提升，而 IR64 中 *PDC2* 的表現量在淹水後第一個小時就達到最高峰，其後表現量便迅速下降。FR13A 中 *PDC2* 基礎表現量是 IR64 的五倍 (Table 2)。

Table 2. Real time RT-PCR basal expression levels

	sample	ΔCt	ΔΔCt	$2^{(-\Delta\Delta CT)}$
ADH1	FR13A	5.86	0.00	1.00
	IR64	4.91	-0.95	1.93
ADH2	FR13A	4.68	0.00	1.00
	IR64	2.73	-1.95	3.86
PDC2	FR13A	-1.48	0.00	1.00
	IR64	1.03	2.51	0.18

Relative expression values are shown with the $2^{-\Delta\Delta Ct}$ method (1=FR13A expression).

FR13A 會限制水溶性碳水化合物及澱粉的消耗但增加 *ADH* 及 *PDC* 的表現量，增加了酒精醱酵的能力。*PDC* 與 *ADH* 跟淹水時的碳水化合物消耗並沒有直接相關 (Fukao et al,. 2006)。Acetaldehyde 在細胞內過量可能導致細胞自身的損害 (Drew et al., 1997; Gibbs and Greenway, 2003)；*ADH* 在 FR13A 中戲劇性的增加可能是因爲需要將大量的 acetaldehyde 迅速轉爲酒精以避免

自身細胞受損 (Kundu et al,. 1993)，而細胞中的 acetaldehyde 的量亦應該因爲先前較高的 *PDC* 表現量而自丙酮酸轉換較多較迅速。較高的 *PDC* 表現量亦被認爲與淹水後的存活有正相關性 (Quimio et al,. 2000)。

一般培植中，IR64 因爲是特別育種過的、高產量又便於耕種的稻系，其生長及抽穗等明顯較 FR13A 還要快出許多。可能也是因爲它快速生長的特性，使得文獻中它在成株時的耐水性不若生長緩慢許多的 FR13A。在其幼苗亦可見到此特性；在淹水 24 小時之後不論在胚鞘的生長或初生根的生長，其表現均優於 FR13A。

大部分的水稻品種在持續淹水一周後就會死亡，但 FR13A 可以在持續淹水 2 個星期後繼續存活。目前發現這樣的差異主要是和一群位於第 9 條染色體著絲點附近的數量遺傳基因座 Submergence 1 (Sub1) 有關 (Fukao et al., 2006)。在 Sub1 基因座中有 3 個基因 Sub1A、Sub1B 和 Sub1C。其中 Sub1B 和 Sub1C 在水稻 2 個亞種 (japonica 和 indica type) 中都存在，但 Sub1A 則有品種間的差異，不具有 Sub1A 基因的水稻品種無法在淹水下存活，而即使具有 Sub1A 基因也分爲 Sub1A-1 (耐淹水) 和 Sub1A-2 (不耐淹水) 2 種不同的基因型。本實驗中所使用的 IR64，含有 Sub1A-2 Sub1C-3、5，並不含 FR13A 的 Sub1A-1 及 Sub1C-1 基因 (Xu et al., 2006)，但在淹水 24 hours 後生長明顯的較 FR13A 優，且負責製造 ADH 或 PDC 的基因並不在於 Sub1，因此對抗淹水並不單經由調節醱酵作用。

四、結論

全球暖化下，淹水是許多稻作產地即將面臨的困境。在受到淹水逆境時，植物的型態、生理將有所改變。雖然植物在淹水時會進行無氧代謝，但長期缺氧下植物並不能存活。本研究的目的即是了解低氧狀態下水稻的基因表現及細胞反應，試圖改善植物對抗水逆境。

水稻 FR13A 因耐水性極佳而常用於分子育種，IR64 產量高卻不耐水。是那些特質及反應使稻種間有不同耐水機制？我們以三天大幼苗，觀察其淹水 24 小時後生長情形、通氣組織的變化及應用即時反轉錄聚合酶鏈反應 (real time RT-PCR)，比較將有氧呼吸轉為酒精醱酵的主要蛋白質：乙醇脫氫酶 (alcohol dehydrogenase, *ADH1*, *2)* 及丙酮酸脫羧酶 (pyruvate decarboxylase, *PDC2*)基因之表現量。

本研究發現 FR13A 的幼苗在淹水時採取抑制自身生長的策略，IR64 則未採取此策略。一般而言良好的通氣組織對於淹水之後 (desubmergence) 生長恢復也是極為重要的關鍵；在耐水性佳的 FR13A 中即可發現其已形成的通氣組織在淹水後，的增加較 IR64 多。

在基因轉錄方面，淹水時 FR13A 的 *ADH1*、*ADH2* 表現量皆被誘導而增加，但表現趨勢不盡相同。FR13A 中 *ADH1* 的表現量在淹水後第一個小時就達到最高峰，而後便逐漸下降，但 IR64 中 *ADH1* 的表現量要在淹水後第二十四小時才有提升。FR13A 中 *PDC2* 的表現量在淹水處理後第二十四小時才出現增加情況，而 IR64 中 *PDC2* 的表現量在淹水後第一個小時就達到最高峰，其後表現量便迅速下降。值得注意的是，IR64 中 *ADH1* 的基礎表現量在對照組 (未受淹水處理) 中的表現量為 FR13A 對照組的兩倍。儘管如此，當 FR13A 受到淹水處理時其 *ADH1* 表現量皆較 IR64 高。

由此可知，FR13A 較佳的耐水性是由於在完全淹水時減緩成長，以保留體內的碳水化合物不受酒精醱酵作用揮霍；並將能量運用在維持體內通氣組織的持續生長。且酒精醱酵中的基因有獨特誘導反應，因此耐水性較佳。藉由這個研究希望能揭開耐淹水稻在淹水時，細胞學及分子生物學上的反應策略，進而改良稻作，使高產量的稻種也能有良好的耐淹水性。

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Supplement

Molecular and Cellular Responses under Hypoxic Stress among Rice Cultivars with Different Flooding Tolerance

缺氧逆境下不同耐淹水性稻子的分子生物及細胞反應

S1. Experiment flow chart p. 2

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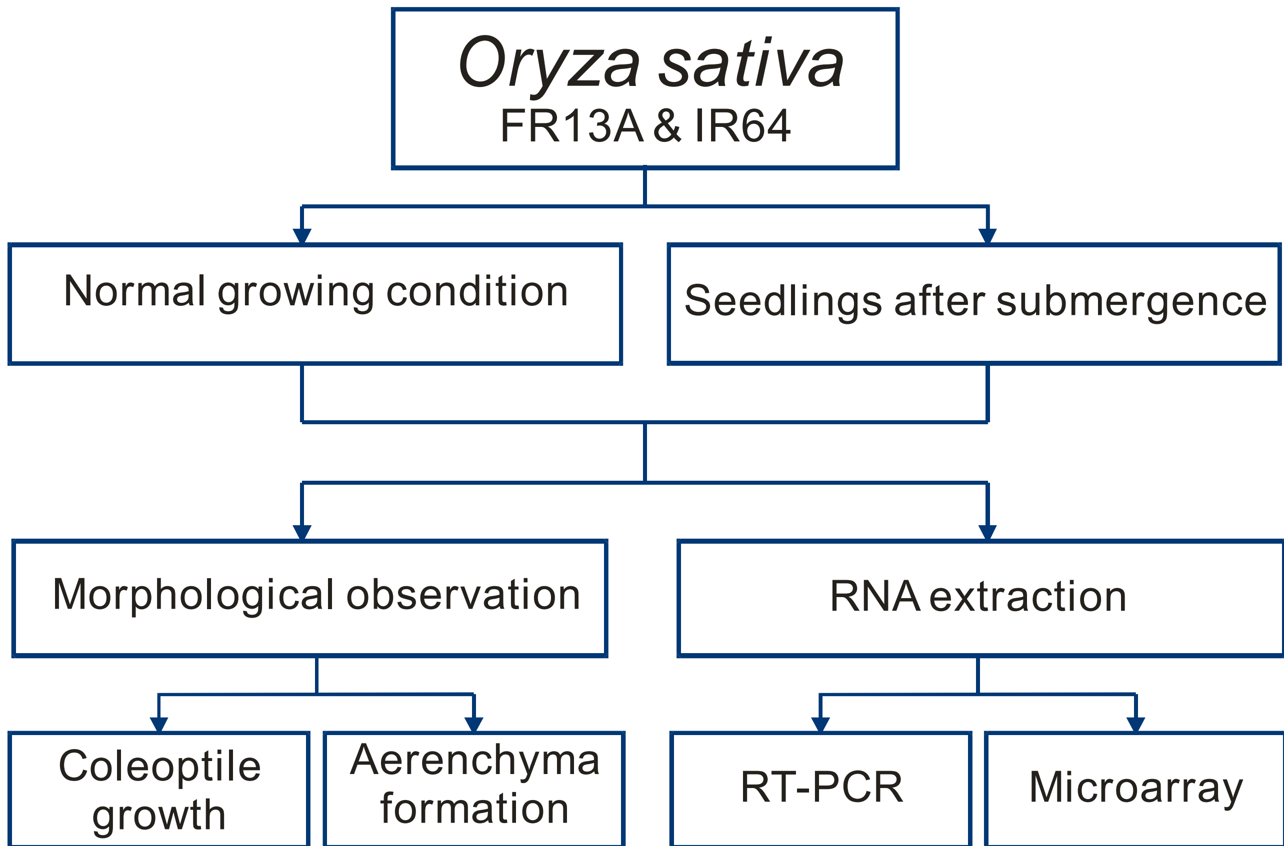
Cluster 3: SUS1 coordinate expressed genes list p. 20

S7. Carbohydrate metabolism pathway with gene expressions p. 22

S1. Experiment flow chart



S4. Pictures of coleoptile growth in submergence

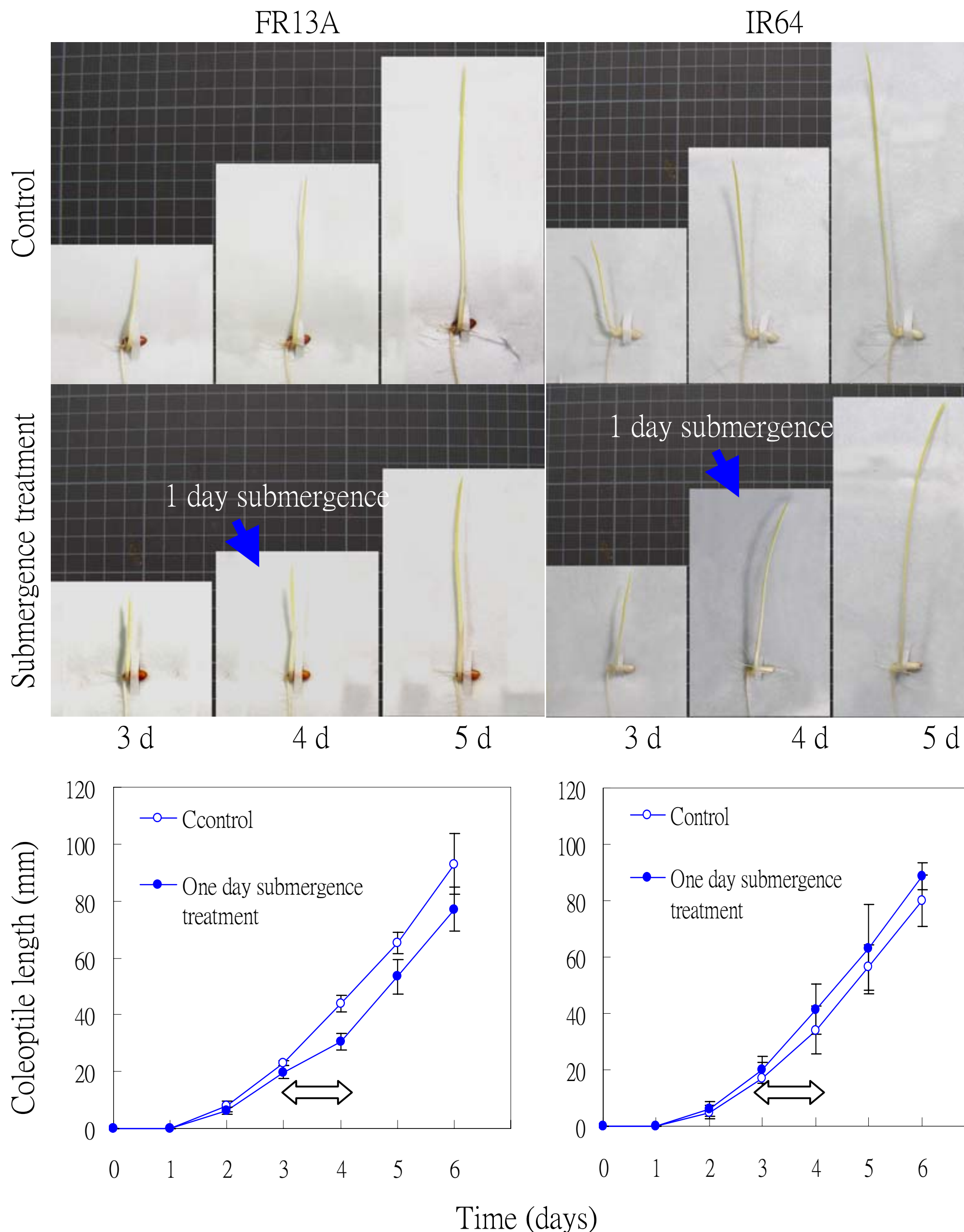


Fig. Coleoptile elongation in FR13A is lesser than in IR64 plants under submergence. Seedlings were germinated on two sheets of moist filter paper in darkness at 30°C in a growth chamber. Three-day-old rice seedlings were then completely submerged for 24 hours (b), (e) and returned to normal growth conditions for another 2 days. The control groups (a), (d) were left in the original condition for the same time duration. The length of coleoptiles (c), (f) was measured with a ruler and photographed everyday. A composite images showed effects of one day hypoxia on coleoptile growth of 2 rice cultivars. The two headed arrow indicates the period of the 24 hours hypoxic treatment. The data represent means ± sd from three independent biological replicates.

S3. Protocol of real time RT_PCR

RNA extraction

1ml Trizol (Invitrogene)
\+ Root samples

Incubate at room temp for 10 min

\+ 200μl chloroform (merk)

Vortex well，Centrifuge for 15min，then transfer 400μl of the aqueous layer to a new centrifuge tube.

100μl isopropanol (merk)
\+ 30μl high salt (1.2M Sodium Citrate, 0.8M NaCl)

Incubate at room temp for 10 min，centrifuge for 10min to precipitate RNA and discard aqueous layer; then wash with 75% alcohol and 100% alcohol and air dry the RNA pellet.

DNase free treatment

21μl RNA
2.45μl 10X DNase Buffer (Turbo DNA-free kit, Ambion)
\+ 1μl DNase (Turbo DNA-free kit, Ambion)

Incubate at 37° C for 30 min.

\+ 2μl DNase Inhibitor

Gently mix for 2 minutes then centrifuge and take 21μl of the aqueous layer.

First strand cDNA synthesis

21μl RNA
2μl oligo-dT
2μl 10mM dNTP
\+ 1.7μl H_2O

Incubate at 65° C for 5 min.

8μl 5XRT buffer (Invitrogen MMLV Reverse Transcriptas
e)
4μl 0.1M DTT
0.3μl RNase out (Invitrogene RNase Out)
\+ 1μl RT

Incubate at 37° C for 15 min then incubate at 70° C for 15 min.

S4. Results of real time RT_PCR

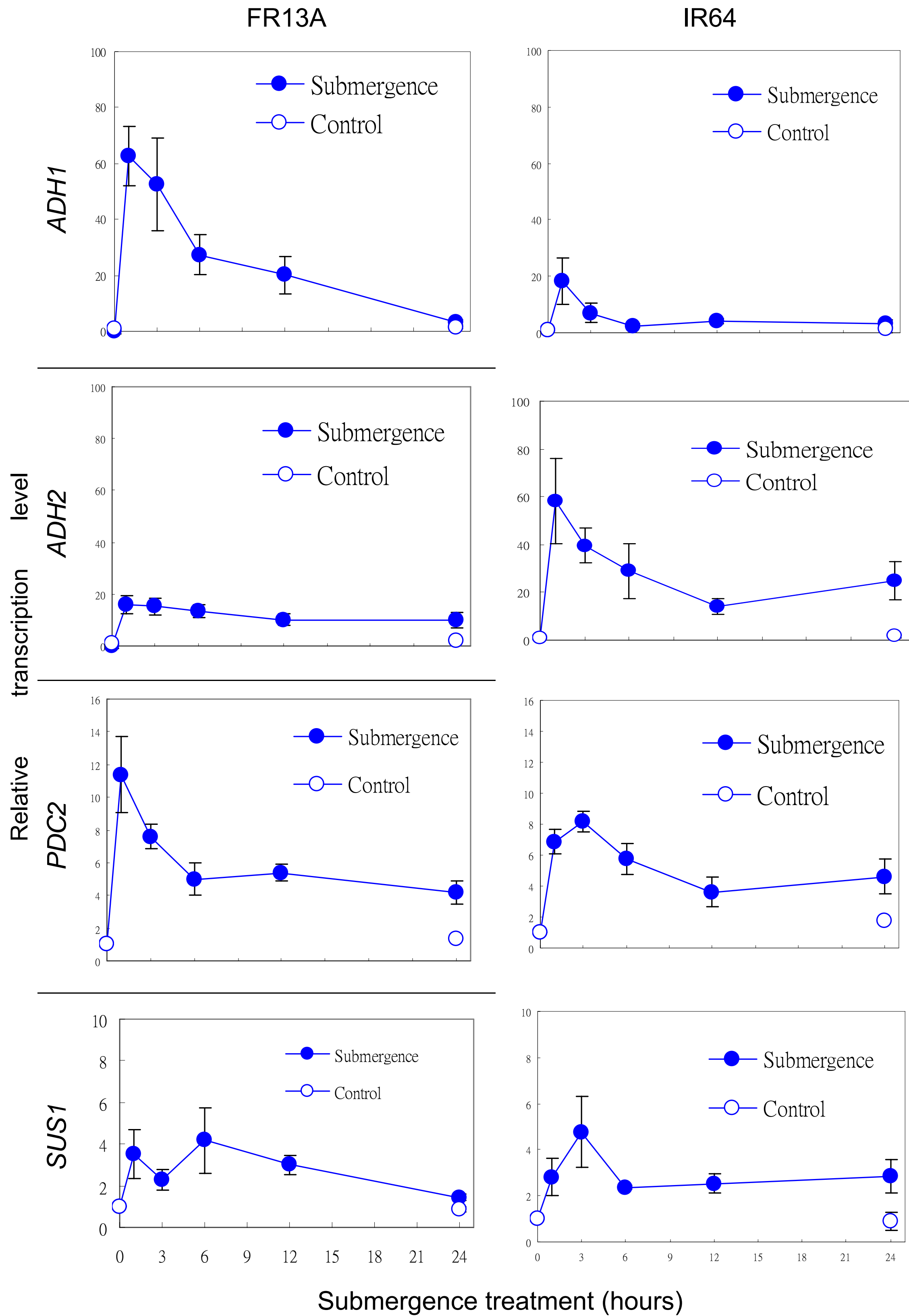


Fig. Effects of submergence on the expression of ADH1, ADH2, PDC2 and SUS2.

Three-day-old seedlings were completely submerged for 1, 3, 6, 12 and 24 hours then root samples were collected to examine the expression pattern of ADH1, ADH2, PDC2 and SUS1. Relative transcription level is shown (1 = expression data of three day old seedlings from the control group); bars are of standard error of mean. In ADH1 and ADH2 expressions, FR13A and IR64 have interestingly adverse expression amounts; however, they all peak in the first hour of submergence. FR13A has a 70 fold expression in ADH1and a ten fold expression in ADH2, while IR64 had a 25 fold expression in ADH1 and a 75 fold expression in ADH2. PDC2 expression is higher in FR13A, peaking by 17 folds in the first hour and remaining at a 7 fold expression through the 24th hour of submergence. IR64 has an evidently smoother curve that slightly peaks by 7 folds in the third hour of submergence, and remaining a 5 fold expression through the 24th hour. FR13A has a sustained 2-fold expression of SUS1 that lasts for at least the first 12 hours of submergence, slightly dropping back to one fold of expression in the 24th hour. IR64 has a more jagged expression curve with peaks of 3~ folds at the 3th and 12th hours of submergence and troughs of 2~ folds at the 6th and 24th hour of submergence. But overall the expression of IR64 SUS1 is much the same as FR13A.

S5. Protocol of Microarray

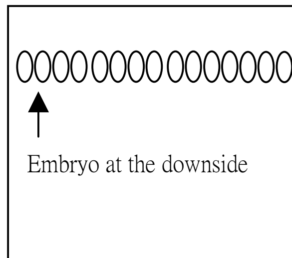
MICROARRAY PROTOCOL

NSF Rice 45K Array

1. Prepare Sample (Day 1 to Day 5)
2. Extract RNA (Day 5)
3. Check RNA quality (Day 6)
4. Synthesize cDNA and Hydrolysis RNA(Day 7)
5. Purify (Day 7) and Label cDNA (Day 8)
6. Hybridization (Day 8)
7. Wash the slide (Day 9)

1. Prepare Sample (Day 1 to Day 5)

1. Place 2 g rice seeds in a 250 ml flask and wash the seeds by (30 ml D.D.W and 20 ml COLAX bleach; optional: add a little bit Tween-20) (* for fewer than 3 hr exp, I use about 6 g seeds for one chip set .)
2. Shake the seeds for 15 min on a shaker or in a shaking incubator.
3. Change the solution and wash again.
4. Use D.D.W to wash out bleach at least 4 times (each time more than 50 ml)
5. Put seeds in a line in 12.5 x 12.5 square plate with two filter papers



(40-50 seeds per plate)

6. Put some water before sealing the plate.
7. Put the plates in a dark incubator at 30℃.

※1. usually germinate seeds Thursday afternoon and collect the root Monday morning. Make sure plant the seeds and collect the sample at the consistent time.

2. 0 hr-3 hr/ one chip set = 3 plate rice roots; 6 hr-12 hr/ one set chip = 4-5 plate rice roots.

* Hypoxia treatment: 3 plates in 1 L Tris-HCl buffer (pH=7.5, final 10 μM)

2. Extract RNA (Day 5)

1. Apply TRIZOL LS solution to a 50 ml tube and preheat it at 50℃ (1g tissue/ 10 ml

TRIZOL LS)

2. Homogenize plant tissues in liquid nitrogen.
3. Add the sample powder into the tube containing pre-heat TRIZOL LS solution, and vortex for 30 sec.
4. Stand at 50℃ for 2 min, then at RT for 5 min and keep it on ice for 1 hr.
5. Add chloroform 0.2 X vol. (2 ml chloroform /10 ml sample volume) and shake vigorously for 1 min. Then leave for 5 min at RT.
6. Centrifuge at 10,000 g at 4 ℃ for 25 min.
7. Take each 1 ml supernatant to each 1.5 ml tube and add 200 µl chloroform.
8. Vortex vigorously and centrifuge at 12,000 g at 4 ℃ for 15 min.
9. Take each 400 µl sample from the aqueous layer into each new 1.5 ml tube and then add 100 µl isopropanol and 30 µl high salt solution (1.2 M Sodium Citrate, 0.8 M NaCl) (Mix well!!)
10. Incubate the sample at room temperature for 10 min.
11. Precipitate the RNA pellet at 4℃ for 12,000 g for 10 min.
12. Wash the pellet with 75% EtOH.
13. Wash the pellet with 100% EtOH.
14. Remove the supernatant, and air dry the pellet about 30 min.
15. Re-suspend the pellet in DNase and RNase free water (Don't use DEPC-treated water, DEPC may influence the RT efficiency!).
16. Concentrate the RNA sample until the concentration more than 5 µg/µl.
17. DNase treatment for 30 min at 37℃
18. Inactive DNase for 2 min at RT. Then, Centrifuge the sample twice at 4℃.
19. Quantify RNA concentration and run 2100 Bioanalyzer.

3. **Check RNA quality (Day 6)**

1. Mix 2 µl RNA (total RNA= 4 µg) with 0.5 µl oligo dT-V (2 µg/µl)
2. Set a PCR machine: heat 10 min at 70℃ and quickly chill on ice for 5 min.
3. Add

5X Superscript II buffer	1 µl
0.1 M DTT	0.5 µl
50X dNTP	0.1 µl
Cy3 dUTP	0.5 µl
Superscript II reverse transcriptase (200 U/µl)	0.4 µl

4. Set a PCR machine: 42℃ for 2 hr
5. Clean up the sample: Add 95µl water to the sample and clean up by QIAGEN PCR clean up kit. Finally, elute the DNA with 50 µl EB and dry down to 4 µl.
6. Mix the sample with 6X DNA Dye.
7. Run a 1% gel (0.15 g agarose powder + 15 ml 1X TBE buffer) for 30 min. At the first 15 min, turn on Typhoon Scanner.

8. Typhoon Scanner: Fluorescence: Cy3 =650; Cy5 =500 (Here just chose Cy3)

Orientation: R

Focus: 3 mm +

4. Synthesize cDNA and Hydrolysis RNA (Day 7)

	Cy5 total RNA	Cy3 total RNA
Oligo dT-V primer	1.25 μl	1.25 μl
Total RNA 35 μg	X μl	X μl
RNase Free Water	X μl	X μl
Final volume	15 μl	15 μl

* Oligo dT-V (2 μg/μl) desalted.

1. Incubate at 70℃ for 10 min. Spin and chill on ice for 10 min.

2. Reverse Transcription (first cDNA synthesis)

Reagent	Amount
Water	3.4 μl
5X superscript buffer (Invitrogen)	6.0 μl
50X aa dUTP/dNTPs (4:1)	0.6 μl
0.1 M DTT	3.0 μl
Total volume	13 μl

3. Add RT reagent and mix well.

4. PCR machine program:

   65℃ 2 min
   42℃ 3 min
   42℃ 60 min
   42℃ 60 min
   4℃ ∞

5. Pause at first 42℃ –60 min and add 1 μl Superscript II (Mix well!!)

6. Pause again at 2nd 42℃ –60 min and add 1 μl Superscript II (Mix well!!)

7. Hydrolysis RNA

Reagent	Amount
0.5 M EDTA	10 μl
1.0 N NaOH	10 μl

8. Add one kind of the reagents, vortex once. (Mix well!!)

9. Incubate for 15 min at 65℃ .

10. Neutralize with 25 μl 1 M HEPES pH=7.4. (Mix well!!)

5. Purify (Day7) and Label cDNA (Day 8)

1. Fill one Microcon-30 concentrator with 375 μl of DDW.

2. Add the neutralized sample (75 μl)(Mix well!!)

3. Spin at 12,000 x g for 10 min
4. Save the first flow through
5. Add 450µl DDW to the column and Spin at 12,000 x g for 10 min- Wash I (Mix Well!!)
6. Discard the flow through, Add 450 µl DDW to the column and Spin at 12,000 x g for 10 min- Wash II (Mix Well!!)
7. Discard the flow through, Add 450 µl DDW to the column and Spin at 12,000 x g for 10 min- Wash III (Mix Well!!)
8. Discard the flow through. Invert and insert the column into a new tube.
9. Spin for 1 min at 1000 x g
10. check the solution volume and make its final volume 50 µl
11. Spin for 3 min at 1000 x g
12. Check the concentration by NaroDrop.(Normal concentration about 20-35 ng/µl)
13. Concentrate the sample to 7 µl by Speed Vac at 30℃.(Make sure not overdry!)
14. Coupling reaction:

Reagent	Amount
Cy3 or Cy5	Dissolve the dry pellet in 2 µl DMSO
Sodium bicarbonate pH=9.0	1 µl
Sample	7 µl
Total volume	10 µl

15. Coupling for 1 hour at 25℃ in the dark.
16. Mix and Spin the coupling sample each 15 min. (total 4 times)
17. Quench Cy Dye by adding 4.5 µl 4 M hydroxylamine . (Mix well!!)
18. Incubate for 15 min at 25℃ in the dark.
19. Remove unincorporated Cy dye by QIAGEN PCR clean up kit.
20. Add 70 µl DDW.
21. Add 500 µl PB. (Mix Well!!)
22. Apply to QIAquick column and spin at 13,200 rpm for 1 min and reapply again.
23. Discard the flow through.
24. Add 750 µl Buffer PE and spin 1 min (repeat 3 times!)
25. Spin at 13,200 rpm for 1 min to dry the column.
26. Transfer the column into a new tube.
27. Add 40 µl Buffer EB for the first elution and incubate for 1 min.
28. Add 30 µl Buffer EB for the second elution.
29. Take 0.5 µl sample to run a gel and scan by Typhoon scanner.
30. Concentrate the sample to 1 µl in a SpeedVac at 30℃ (Be sure not overdry!)
    (Combine the Cy3 and Cy5 sample during the volume about 10-20µl !!)

6. Hybridization (Day 8)

Hybridization Buffer:

Formamide 100%	50 µl
20X SSC	25 µl
10% SDS	1 µl
0.1 M DTT	10 µl
DDW	14 µl

1. Hybridization buffer total volume =100 µl; just add 89.6 µl hybridization buffer into the sample.
2. Mix well and incubate at 42℃ for a while.
3. Add 6.4 µl Salmon Sperm DNA
4. Mix well and incubate at 95℃ for 3 min.
5. Centrifuge at 13,200 rpm for 1 min.
6. Put the sample at 42℃ and wait for loading.
7. Slide pre-hybridization:

   a: prepare pre-hybridization buffer

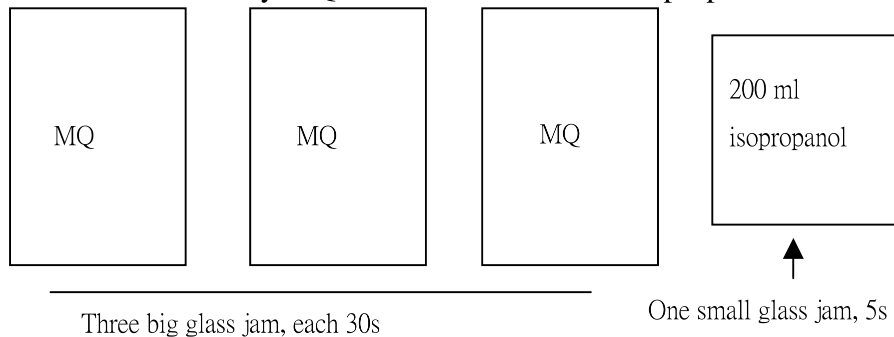
Reagent	Amount
20X SSC	50 ml
10% SDS	2 ml
BSA	Dissolve 2 g BSA in 50 ml water and filter the BSA solution by 0.22µm filter.
Total volume	200 ml

   * Add 10% SDS after the total volume more than 190 ml.

   b: pre-heat the solution at 42℃. (Take more than 2 hour!)

   c: place slides into the pre-hybridization buffer for 45 min (mix each 15 min)

   d: wash the slides by MQ water three times and isopropanol once.



   e: dry the slides for 10 min (turn on the Vac 1 hr earlier!)

8. Pre-heat the Slides in MAUI for 5 min.
9. Seal the Slides with MAUI mixer.
10. Load 48 µl sample into each chip.
11. Hybridize for 18 h at 42℃.

*MAUI takes 2-3 hours to stabilize the temperature. Make sure turn on MAUI earlier!

*MAUI : A mode

7. Wash the slide (Day 9)

1. Sol I (2X SSC, 0.1% SDS)

Reagent	Amount
20X SSC	150 ml
10% SDS	15 ml
Total volume	1500 ml

* Sol I needs to be preheated at 42℃. It takes about more than 3 hours!!

Sol II (0.1X SSC, 0.1 % SDS)

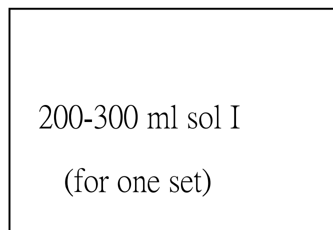
Reagent	Amount
20X SSC	2.5 ml
10% SDS	5 ml
Total volume	500 ml

Sol III (0.1X SSC)

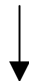
Reagent	Amount
20X SSC	2.5 ml
Total volume	500 ml

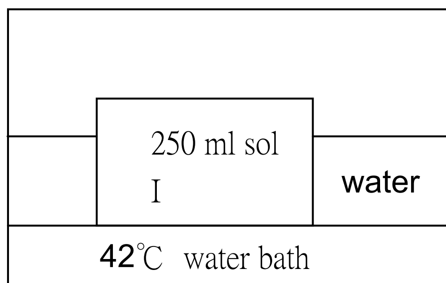
Sol Ⅵ (0.05X SSC)

Reagent	Amount
20X SSC	1.25 ml
Total volume	500 ml

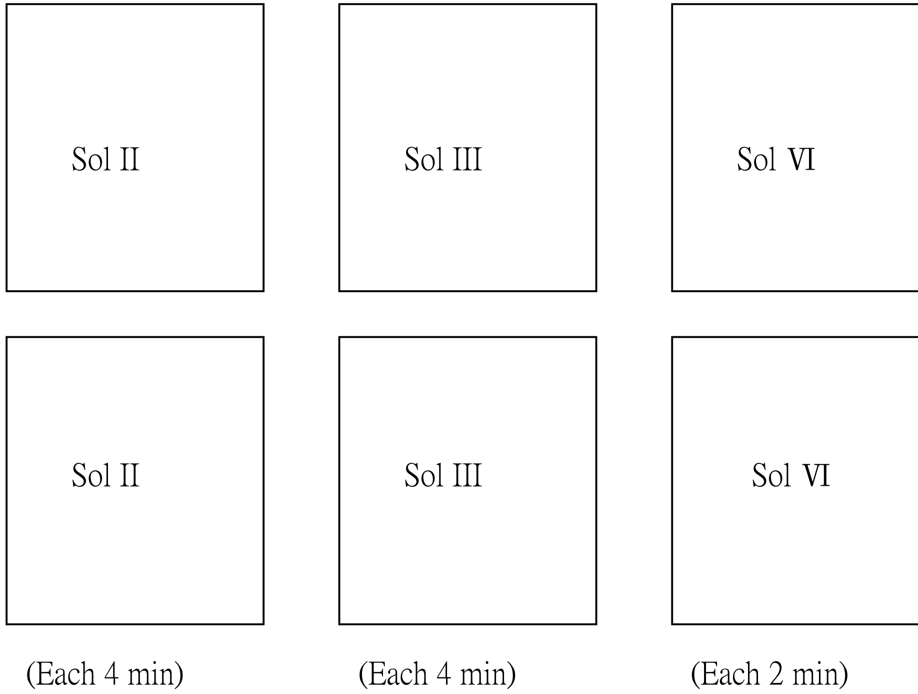


Take out the mixer at Sol I





Wash 4 min (Repeat 3 times, each time replace a new jam)



(250 ml in each jam)

2. dry the slides for 10 min (turn on the Vac 1 hr earlier!)
3. Use Axon Scanner to scan the slides.

S6. Results of Microarray

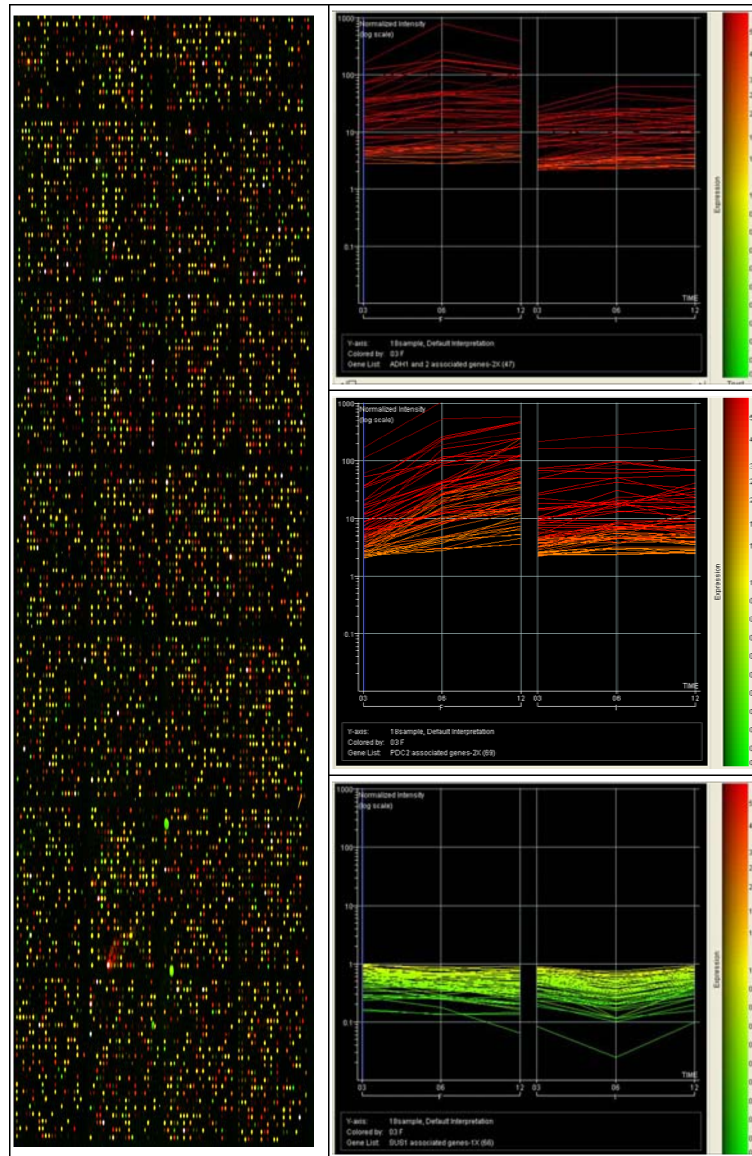


Fig. Representation of expression changes for differentially expressed genes.
Temporal patterns of expression in FR13A and IR64 rice seedlings during the time course of hypoxic stress (3 h, 6 h, and 12 h) were
visualized. Cluster 1 of ADH associated genes showed a rapid increase of gene expression from 0 to 6 h of low-oxygen stress. Clusters
2 of PDC associated genes showed steady increased expression from 3 h to 12 h of hypoxic treatment. Cluster 3 contained SUS1
associated genes that showed a strong decrease in expression during hypoxic condition. The color of each element in the clusters
represents the expression level of each gene with green representing down regulation and red representing up regulation.

Cluster1: Coordinated gene cluster with ADH1and ADH2

Vegetative cell wall protein gp1 precursor, putative, expressed
Transposon protein, putative, unclassified, expressed
17.4 kDa class I heat shock protein 3, putative, expressed
Multidrug resistance-associated protein 4, putative, expressed
Ethylene responsive element binding protein, putative, expressed
Alcohol dehydrogenase 2, putative, expressed
D-mannose binding lectin family protein, expressed;D-mannose
binding lectin family protein, expressed
Alcohol dehydrogenase 1, putative, expressed
Protein kinase domain containing protein, expressed
N/A
Expressed protein
Expressed protein
Receptor-like protein kinase precursor, putative, expressed
Ubiquitin, putative, expressed
MTD1, putative, expressed
Expressed protein
CXC domain containing TSO1-like protein 1, putative, expressed
Expressed protein
Membrane steroid-binding protein 1, putative, expressed
PX domain containing protein, expressed
Serine/threonine-protein kinase BRI1-like 1 precursor, putative, expressed
Myb-like DNA-binding domain, SHAQKYF class family protein, expressed
N/A
Chlorophyll a-b binding protein 2, chloroplast precursor, putative, expressed
Pollen proteins Ole e I family protein, expressed
Serine--glyoxylate aminotransferase, putative, expressed
UBX domain-containing protein 1, putative, expressed
N/A
Myb-like DNA-binding domain, SHAQKYF class family protein, expressed
Ids4-like protein, putative, expressed
Zinc-finger protein 1, putative, expressed
Expressed protein
DNA binding protein, putative, expressed
Expressed protein
Transparent testa 12 protein, putative, expressed

F-box domain containing protein, expressed
Gibberellin 20 oxidase 2, putative, expressed
Ethylene receptor, putative, expressed
Expressed protein
Transferase, putative, expressed
N/A
ADH1 and ADH2 associated genes-2X P2/2
Expressed protein
NAC domain-containing protein 18, putative, expressed
Typical P-type R2R3 Myb protein, putative, expressed
Expressed protein
Histone H1, putative, expressed
Expressed protein

Cluster 2: Coordinated gene cluster with PDC

N/A
Domain found in Dishevelled, Egl-10, and Pleckstrin family protein, expressed
Expressed protein
Receptor protein kinase CRINKLY4 precursor, putative, expressed
OsWRKY1v2 - Superfamily of rice TFs having WRKY and zinc finger domains, expressed
Expressed protein
N/A
Expressed protein
Spotted leaf protein 11, putative, expressed
F-box domain containing protein, expressed
Expressed protein
Trehalose-6-phosphate synthase, putative, expressed
Pyruvate decarboxylase isozyme 2, putative, expressed
Non-symbiotic hemoglobin 2, putative, expressed
Expressed protein
Catalytic/ hydrolase, putative, expressed
Glutamate decarboxylase, putative, expressed
N/A
Expressed protein
Protein binding protein, putative, expressed
Elicitor-responsive protein 3, putative, expressed
N-acetylglucosaminyltransferase III, putative, expressed
Dehydration-responsive element-binding protein 2D, putative, expressed
KI domain interacting kinase 1, putative, expressed
Serine/threonine-protein kinase receptor precursor, putative, expressed
Expressed protein
Expressed protein
N/A
N/A
Armadillo repeat-containing protein, putative, expressed
ACS-like protein, putative, expressed
DNA binding protein, putative, expressed
Cis-zeatin O-glucosyltransferase 1, putative, expressed
ANAC075, putative,

expressed
Expressed protein
Hypothetical protein
N/A
P0421H07.29 [Oryza sativa (japonica cultivar-group)]
ATP binding protein, putative, expressed
N/A
Universal stress protein, putative, expressed
Bromodomain associated family protein, expressed
Expressed protein
PDC2 associated genes-2X 2/2
Expressed protein
Acetyltransferase, GNAT family protein, expressed
Wound induced protein, putative, expressed
Hypothetical protein
Expressed protein
Lipase precursor, putative, expressed
SPF1-like DNA-binding protein, putative, expressed
N/A
Expressed protein
Expressed protein
DNA-binding protein, putative, expressed
PDC2 associated genes-2X 3/3
Phosphoglycerate kinase, cytosolic, putative, expressed
Respiratory burst oxidase, putative, expressed
Protein binding protein, putative, expressed
Clathrin assembly protein, putative, expressed
Expressed protein
Protein kinase KIPK, putative, expressed
Protein translation factor SUI1 homolog 2, putative, expressed
Transposon protein, putative, unclassified
Expressed protein
Expressed protein
Expressed protein
Histone deacetylase, putative, expressed
Resistance protein LR10, putative, expressed

Chlorophyll a-b binding protein 1, chloroplast precursor, putative, expressed
Calcium-binding mitochondrial carrier F55A11.4, putative, expressed

Cluster 3: Coordinated gene cluster with SUS1

CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase, putative, expressed
Malate dehydrogenase, mitochondrial precursor, putative, expressed
Outer membrane protein, OMP85 family protein, expressed
Catalytic/ protein phosphatase type 2C/ protein serine/threonine phosphatase, putative, expressed
Nucleic acid binding protein, putative, expressed
IAP100, putative, expressed
Tumor-related protein-like, putative, expressed
DNA binding protein, putative, expressed
Coatomer subunit epsilon, putative
Phytosulfokine receptor precursor, putative, expressed
Auxin response factor 2, putative, expressed
MAP3K-like protein kinase, putative, expressed
Pathogenesis-related protein PR-1 precursor, putative, expressed
Expressed protein
Proteasome subunit alpha type 5, putative, expressed
Actin polymerization factor, putative, expressed
Elongation factor 1-gamma 3, putative, expressed
Nucleotide-binding protein 1, putative, expressed
Expressed protein;expressed protein;expressed protein
Lysine ketoglutarate reductase trans-splicing related 1, putative, expressed
Protein arginine N-methyltransferase 6, putative, expressed
Ribosome recycling factor, chloroplast precursor, putative, expressed
Calmodulin binding protein, putative, expressed
Expressed protein
UBA and UBX domain-containing protein, putative, expressed
Protein G10 homolog 2, putative, expressed
Haloacid dehalogenase-like hydrolase domain-containing protein 1A, putative, expressed
Nucleoside-diphosphate-sugar epimerase, putative, expressed
Expressed protein

Chaperonin, chloroplast precursor, putative, expressed
Ferredoxin-6, chloroplast precursor, putative, expressed
Acetylglutamate kinase, putative, expressed
Rac-like GTP-binding protein 2, putative, expressed
Expressed protein
Tic20-like protein, putative, expressed
Sugar transport protein 5, putative, expressed
Expressed protein
Tyrosine aminotransferase, putative, expressed
Acyl-activating enzyme 18, putative, expressed
Expressed protein
Expressed protein
Thiosulfate sulfurtransferase, putative, expressed
Expressed protein
SUS1 associated genes-1X 2/2
Adagio protein 1, putative, expressed
PHD finger protein, putative, expressed
YGR159c, putative, expressed
Integral membrane protein like, putative, expressed
Glutathione synthetase, chloroplast precursor, putative, expressed
Proline-rich protein, putative, expressed
Expressed protein
Dihydrolipoyllysine-residue acetyltransferase component of pyruvatedehydrogenase
complex, mitochondrial precursor, putative, expressed
Ubiquitin-protein ligase, putative, expressed
Cytochrome b5 isoform 2, putative, expressed
Nodulin-like protein, putative, expressed
Permease, putative, expressed
Cyclin delta-2, putative, expressed
Sucrose synthase 1, putative, expressed
Zeta-carotene desaturase, chloroplast precursor, putative, expressed
Expressed protein
Lysyl-tRNA synthetase, putative, expressed
RING finger and CHY zinc finger domain-containing protein 1, putative, expressed
Sulfate transporter 1.2, putative, expressed
Exosome component 10, putative, expressed
Integral membrane protein like, putative, expressed
Chloroplast 30S ribosomal protein S10, putative, expressed
Importin alpha-1b subunit, putative, expressed

S7. Carbohydrate metabolism pathway with gene expressions

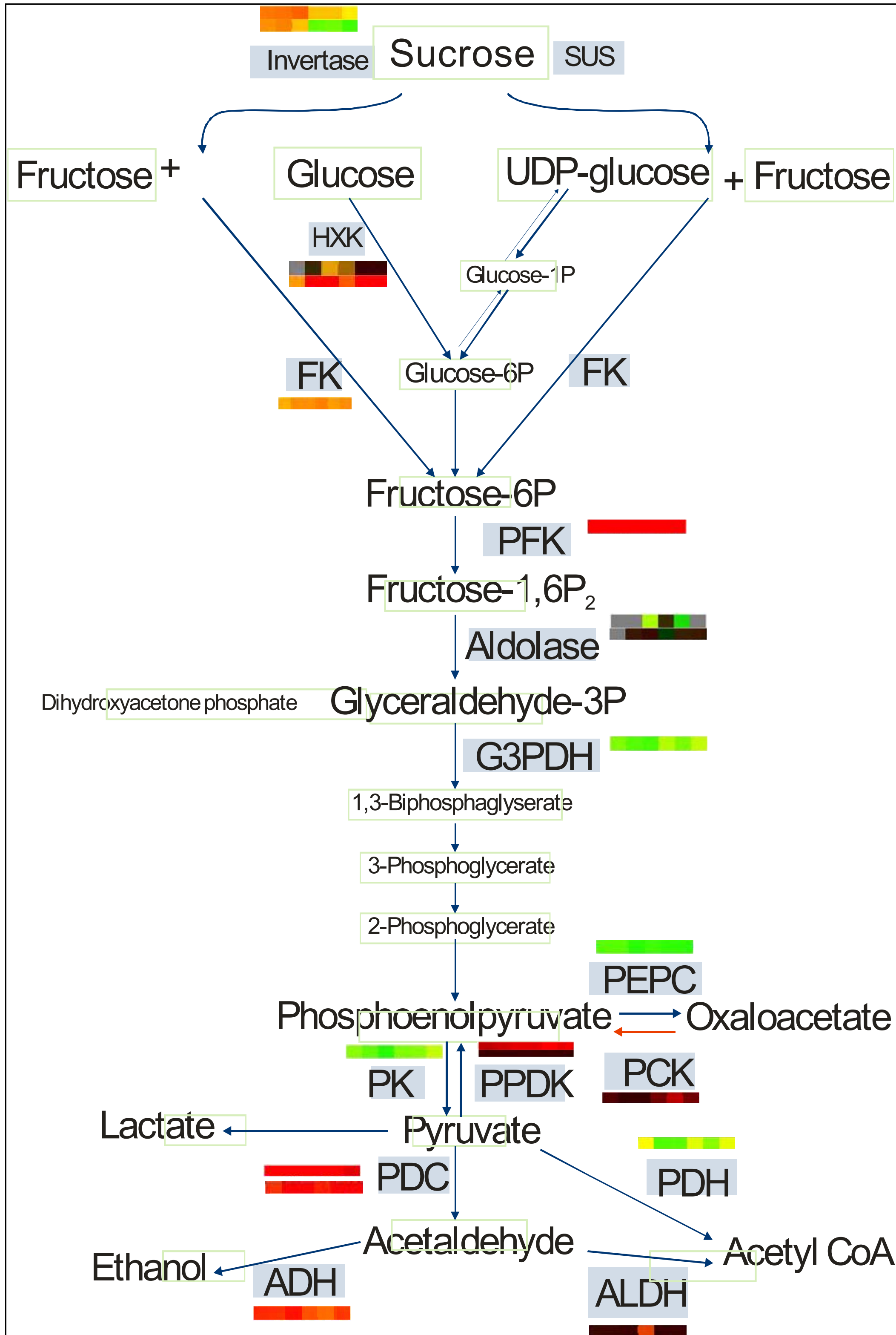


Fig. Effects of hypoxia on carbohydrate metabolism in rice seedlings roots.
Data mining of the transcriptome of hypoxic seedlings roots allowed the identification of the genes involved in sucrose metabolism and glycolysis. Genes showing a statistically significant change in expression when the aerobic dataset was compared with the anoxic dataset are reported on the metabolic pathway shown in this figure. Red arrows highlight the metabolic steps that, based on transcripts level changes, are strongly up-regulated under anoxia, whereas blue arrows indicate down-regulation. Abbreviations are as follows: ADH, alcohol dehydrogenase; ALDH, acetaldehyde dehydrogenase; FK, fructokinase; G3PDH, NADP-dependent Glyceraldehyde-3-phosphate dehydrogenase; HXK, hexokinase; PCK, phosphenolpyruvate carboxylase kinase; PDC, pyruvate decarboxylase; PDH, pyruvate dehydrogenase; PEPC, phosphenolpyruvate carboxylase; PFK, ATP-dependent phosphofructokinase; PK, pyruvate kinase; PPDK, pyruvate Pi dikinase; SUS, sucrose synthase.

評語

非常有潛力的作品，作者的中、英文表達能力都有一流的表現，爲求作品更加完整，建議作以下的改進。

1) 調整結果呈現的順序。

2) 加強生化及生理的探討，以更進一步支持所提之能量保存的理論。

3) 根部切面的取樣位置，應作調整。

Molecular and Cellular Responses under Hypoxic Stress in Rice Cultivars with Different Flooding Tolerance

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Kaohsiung Municipal Girl's Senior High School, Kaohsiung, CHINESE TAIPEI

Introduction

Global warming results in rising sea levels, increases in occurrence and severity of storms and flooding in river flood plains and arable farmland, battering the south and southeast Asia, where 70% of the world's poor live (IPCC, 2007). Flooding causes a loss of up to 100% of rice harvest, which is their staple. There has been observation that some crop cultivars can withstand flooding stress, thus raising the question: what in particular causes variations of submergence tolerance within different cultivars?

Known responses of plants under submergence include growth rate change, aerenchyma formation, an initiation of fermentation and other changes within the energy yielding processes.

Many reviews have distributed the syndromes into two kinds of strategies: the escape strategy and the quiescence strategy. The escape strategy stimulates the plant's elongation rate, which can restore contact between leaves and air, but can also result in plant death if the plant has not elongated enough to emerge from the water before the depletion of energy. The other strategy in contrast is the quiescence strategy, which slackens the plant's growth and metabolism, reserving energy for growth post-submergence(Perata & Voesenek, 2007). The latter method is more suitable for flash-floodings or long-term floodings.

Morphological adaption to low oxygen stress include the formation of aerenchyma, root cortical air spaces that promote air transport from shoot to root (vartapetian and Jackson, 1997). Aerenchyma formation within rice is intrinsic (Visser et al., 2004: Colmer, 2003). There are two main types of aerenchyma: aerenchyma formed by cells parting is named as schizogenous aerenchyma; aerenchyma formed by cell lysis is known as lysigenous aerenchyma. Rice aerenchyma belongs to the latter (Seago JR et al., 2005). Lysigenous aerenchyma is formed via programmed cell death, thus ethylene participation is needed.

As for metabolic adaptions to anaerobiosis, the induction of fermentation pathway enzymes (Kennedy et al., 1992) is yet another well known trait. Fermentation under lack of O_2 is crucial for regenerating NAD^+, for sustainment of glycolysis. A crisis in ATP availability ensues because glycolysis is inefficient, yielding 2 to 4 mol ATP per mol hexose as compared with 30 to 36 mol ATP by the mitochondrial electron transport chain. Evaluation of gene transcripts, enzymes, and metabolites in a variety of species and genotypes demonstrated the production of minor metabolic end products that are also important for NAD^+ and $NAD(P)^+$ regeneration.

Although mutant analyses with several species have demonstrated that glycolysis and fermentation are necessary for cell survival under O_2 deprivation, the enhancement of these processes is not well correlated with prolonged endurance of this stress (Drew MC. 1997; Gibbs J,

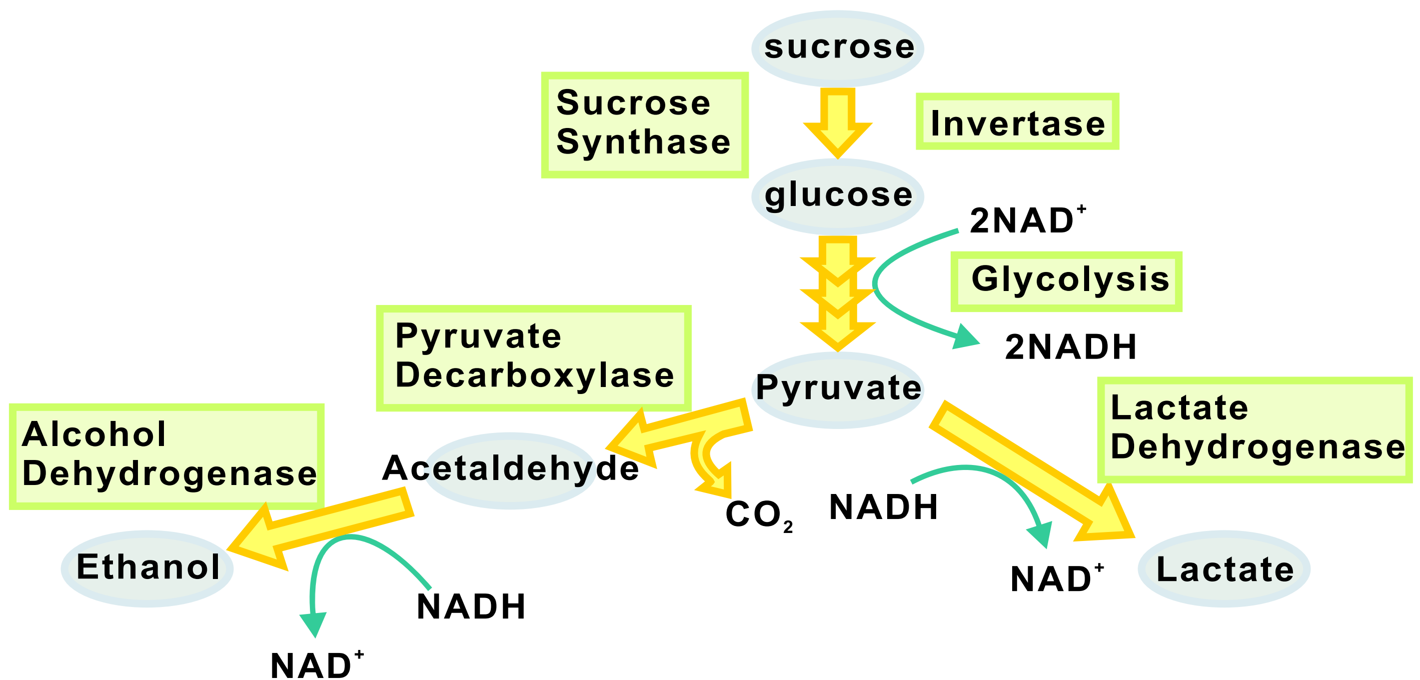


Fig 1. Major products of carbohydrate metabolism in flooded roots.
Pyruvate produced by glycolysis is initially fermented to lactate during the early phase of oxygen distress. Proton production by glycolysis will decrease the cytosolic pH. At lower pH, lactate dehydrogenase (LDL) activity is inhibited, and pyruvate decarboxylase (PDC) is activated. As a consequence of lower pH, the fermentation to ethanol increase and to lactate decrease. The pathway of ethanol fermentation uses up more protons than does the pathway of lactate fermentation. The cytosolic pH will gradually increase and this will enhanced the ability of the plant to cope with the episode of anoxia. (Figure drawn by author)

Greenway H. 2003). Thus cells reliant on externalO_2 limit processes that are highly energy consumptive and alter metabolism to increase anaerobic generation of ATP by cytosolic glycolysis (Drew MC. 1997).

The positive regulation of the SUS route and the repression of INV gene expression and enzymatic activity under O_2 deprivation have been well documented for a variety of plant species and organs including cereal seeds (Guglielminetti et al. 1997), rice seedlings, maize roots (Zeng et al. 1998) and potato roots (Biemelt et al. 1999). In many non-photosynthetic tissues, SuSy exists at high levels in the cytoplasm (Xu et al. 1989; Geigenberger and Stitt 1993), where the products of its activity may be used as precursors in glycolytic metabolism and for synthesis of storage and structural polymers (Winter and Huber 2000). It is assumed to be driven by sucrose starvation (Loreti E et al, 2005).

Thus, this project has focused on the morphological and molecular adaptaions of rice under submergence, including growth change, aerenchyma formation, gene regulation of fermentation and sucrose degradation genes, and gene expression differences on a larger scope including glycolysis.

Materials and methods

Seedlings of FR13A and IR64 rice (Oryza sativa L.) types were analyzed in the course of this study. The seeds were washed vigorously in tap water followed by 1.0% sodium hypochlorite treatment for 5 min. The seeds were subsequently affixed on 2 layer of absorbent 3M sterile filter paper placed in acrylic board and allowed to germinate at 30.0 °C in complete dark culture chamber. 3-day-old seedlings were subjected to 24 hours of submergence in distilled water.

coleoptile growth measurement

The coleoptile length were measured every day, before and after submergence.

aerenchyma formation determination

Aerenchyma change in the free hand section of the roots after 24 hours submergence or in normal (unsubmerged) conditions were examined with a light microscope (Leica model DME) and assayed by Adobe Photoshop CS2.

Molecular observations

RNA samples from the roots used for microarray and real time RT-PCR experiments were extracted from three independent biological replicates.

RNA extraction

Trizol (Invitrogene) was added to root samples then incubated at room temp for 10 minutes before chloroform (merk) was added. After vortexing and centrifuging, the aqueous layer was transfered to a new centrifuge tube. Isopropanol and high salt (1.2M Sodium Citrate, 0.8M NaCl) were added, then the samples were incubated at room temp for 10 min then centrifuged for 10min to precipitate RNA. The aqueous layer is then discarded and the RNA pellet washed with 75% alcohol and 100% alcohol, then air dried. They were cleaned by Turbo DNA-free kit (Ambion) before use.

First strand cDNA synthesis

RNA samples were incubated with oligo-dT and dNTP at 65∘C for 5 min, then 5XRT buffer (Invitrogen MMLV Reverse Transcriptase), DTT, RNase out (Invitrogene RNase Out) and reverse transcriptase was added and the mixture incubated at 37∘C for 15 min then at 70∘C for 15 min.

Real time reverse transcript polymerase chain reaction

RT-PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) in a 7500 Real-Time PCR System (Applied Biosystems). Sequences of interest and corresponding primers are listed in appendix.

Microarray

Cy5- and Cy3-labeled cDNA were pooled and hybridized to NSF 45K Rice oligonucleotide Arrays by using MAUI hybridization system (BioMicro). The hybridization signals for each DNA element were acquired with the use of GenePix 4000B and analyzed with GenePix Pro 6.1 (MDS Analytical Technologies). Microarray data files were imported into GeneSpring GX 7.3.1 (Agilent technologies) for further analyses.

Enzyme activities

Visualization of enzyme activity was by using nondenaturating native-PAGE. Native gel contains 6% acrylamide/bis, 0.5X TBE and 3.125% glycerol. Reaction mixture for ADH contains 0.1M tris buffer (pH 7.5), NAD+ 3mg/ml, MTT 0.2mg/ml, PMS 0.4mg/ml and EtOH 0.6mg/ml.

Results and discussion

Growth adaptation and aerenchyma formation

Coleoptile growth of FR13A under submergence was significantly retarded with 53.8% of the normal condition whereas IR64 had 106% growth of the normal condition. Comparitively, FR13A seems to have taken a "quiscence" strategy, in which the growth is slowered to reduce unnecessary energy consumption (Das et al., 2005). IR64 is a strand that was bred not for abiotic-stress-tolerance but more for high yield and convenient farming; the growth rate and life cycle can be observed to be obviously higher. This trait is precisely the opposite of the quiescence strategy that is suitable for long term flooding, which may cause its tolerance to be weaker than the slow-maturing FR13A.

The formation of aerenchyma in FR13A under submergence increased in approximately the same degree (88.5%) as the aerated group, but was suppressed in the sensitive IR64 (72.2%). Aerenchyma formation within rice is inherent even under normal condtions i.e. no flooding of any part of the plant, thus it is able to present the aerenchyma formation changes by percentages of the original aerenchyma formation. Acknowledged to be

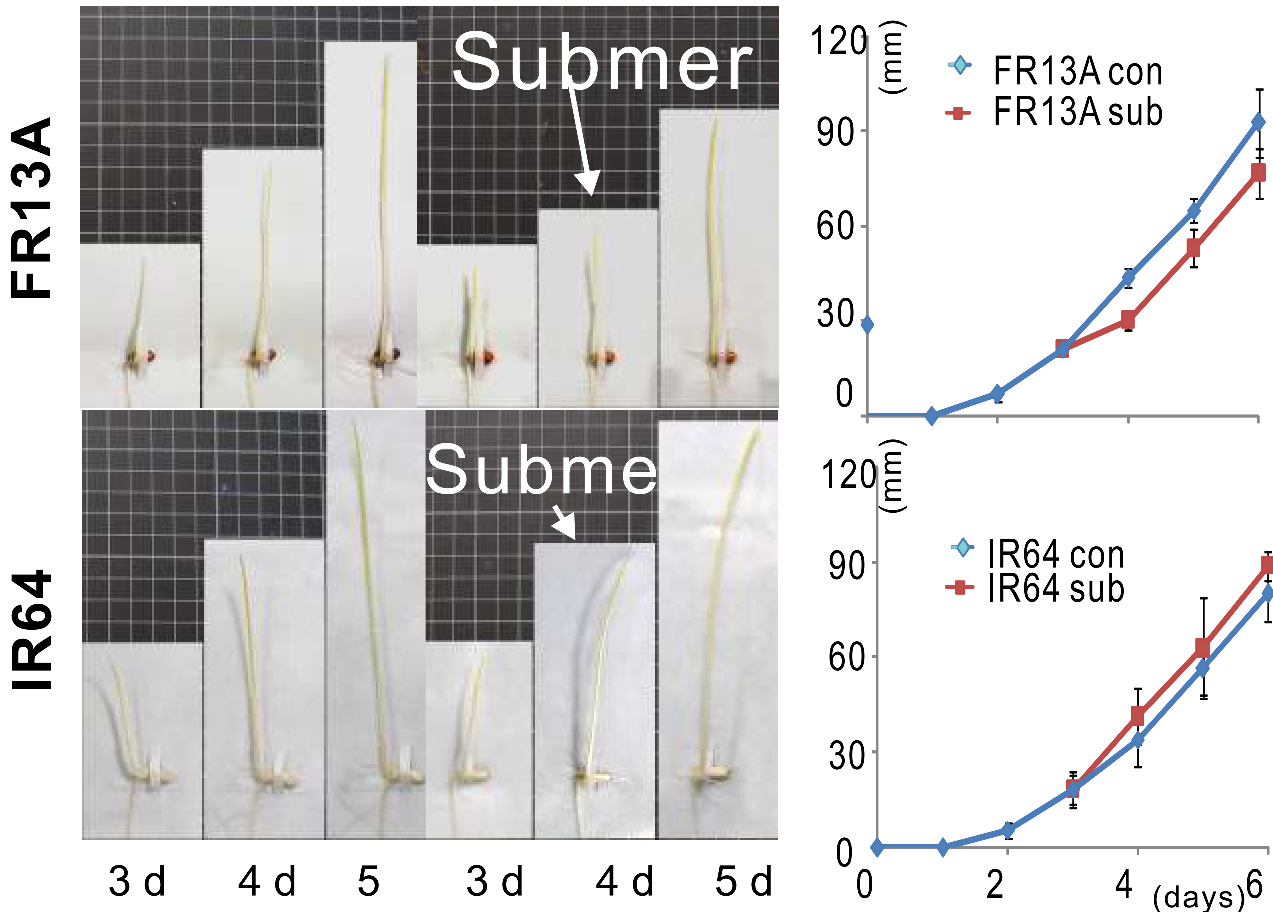


Fig 2. Coleoptile Elongation during Submergence.
Three-day-old seedlings subjected to 24hr submergence were then returned to normal growth conditions for another 2 days and examined for coleoptile elongation. The red line represents the growth of seedlings after submergence treatment. The data represents means ± sd from three independent biological replicates. Coleoptile growth of FR13A under submergence was 53.8% of the normal condition whereas IR64 had 106% growth of the normal condition.

an important indication of flood tolerance within plants, this morphological adaptation may be one of the key features contributing to submergence tolerance in FR13A (Colmer, 2003).

The upstream and downstream of glycolysis

The importance of the fermentation genes (ADH, PDC) is that during O2 deprivation, fermentation is necessary to regenerate NAD+ to sustain glycolysis. Both lactate- and ethanol-producing fermentation yield NADH+. Lactate lowers cytosolic PH, while ethanol does not and can diffuse across plasma membranes. According to the Davis Roberts Lactate dehydrogenase / pyruvate decarboxylase pH-stat hypothesis,

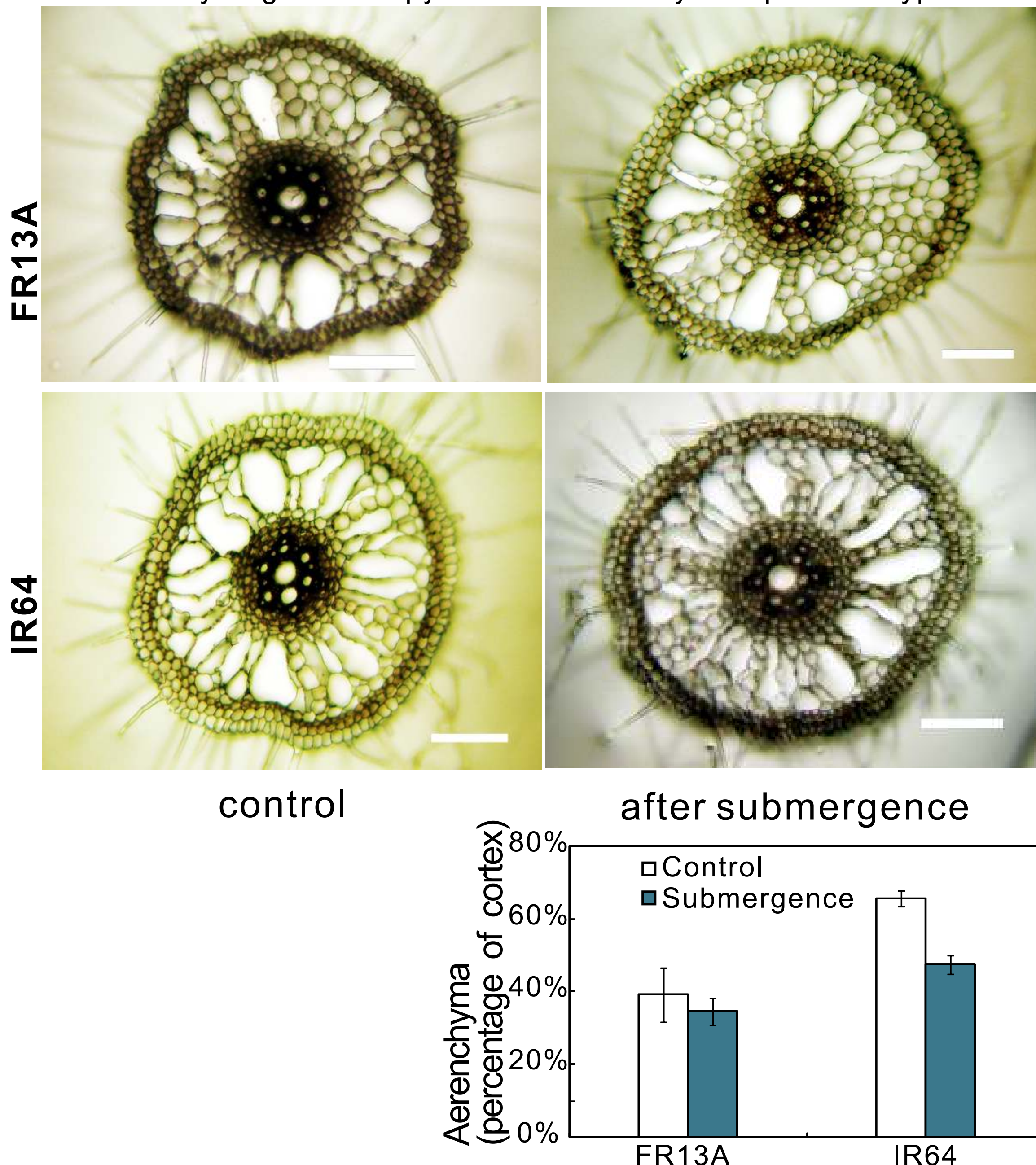


Fig 3. Aerenchyma Change during submergence
Data are means of 24 replicates with standard deviation. (*** $P < 0.001$). The formation of aerenchyma in FR13A under submergence increased in approximately the same degree (88.5%) as the aerated group, but was suppressed in the sensitive IR64 (72.2%) (Fig. 2).

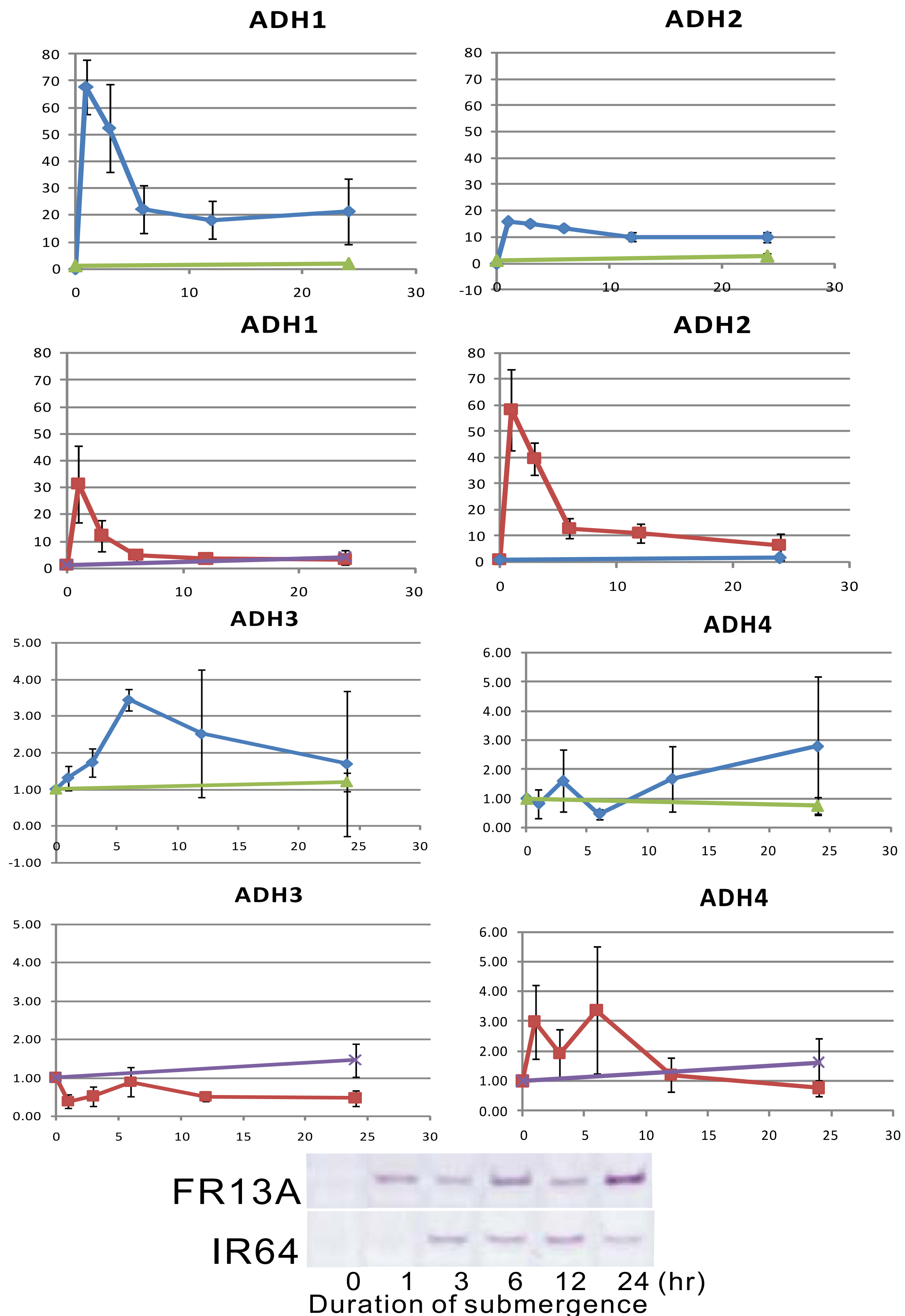


Fig 4. Real-time RT-PCR of *ADH* gene expression and zymography of enzyme activity.
(a)Three-day-old seedlings were submerged for 1, 3, 6, 12 and 24 hours; expression pattern of *ADH1*, *ADH2*, *ADH3* and *ADH4* were examined. Relative transcription level is shown (1 = expression of seedlings from the control group); bars are of standard error of mean. (b) A native-PAGE was conducted with the same time course but isozymes were unable to separate. Enzyme activities of ADH was prominent within FR13A after submergence.

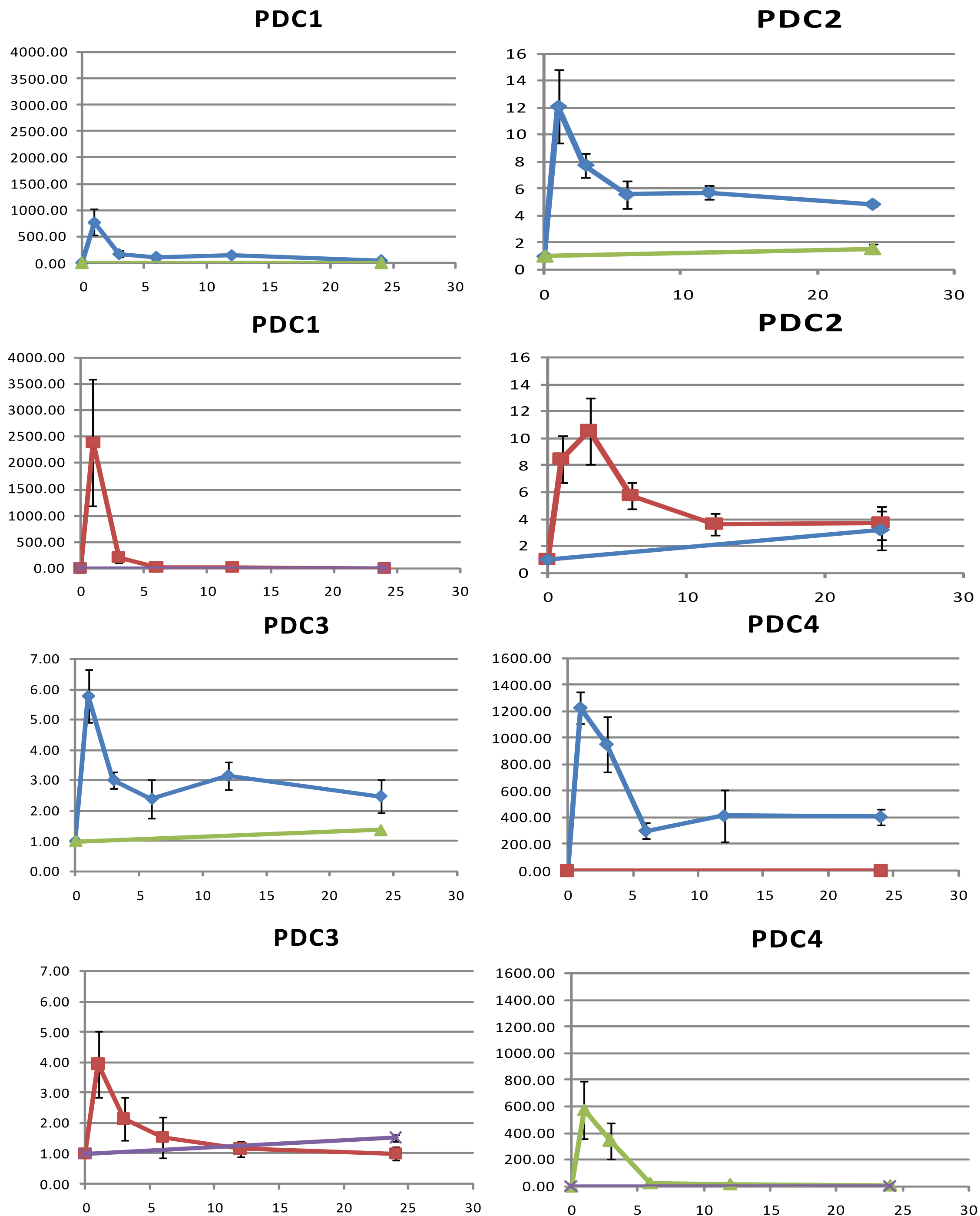
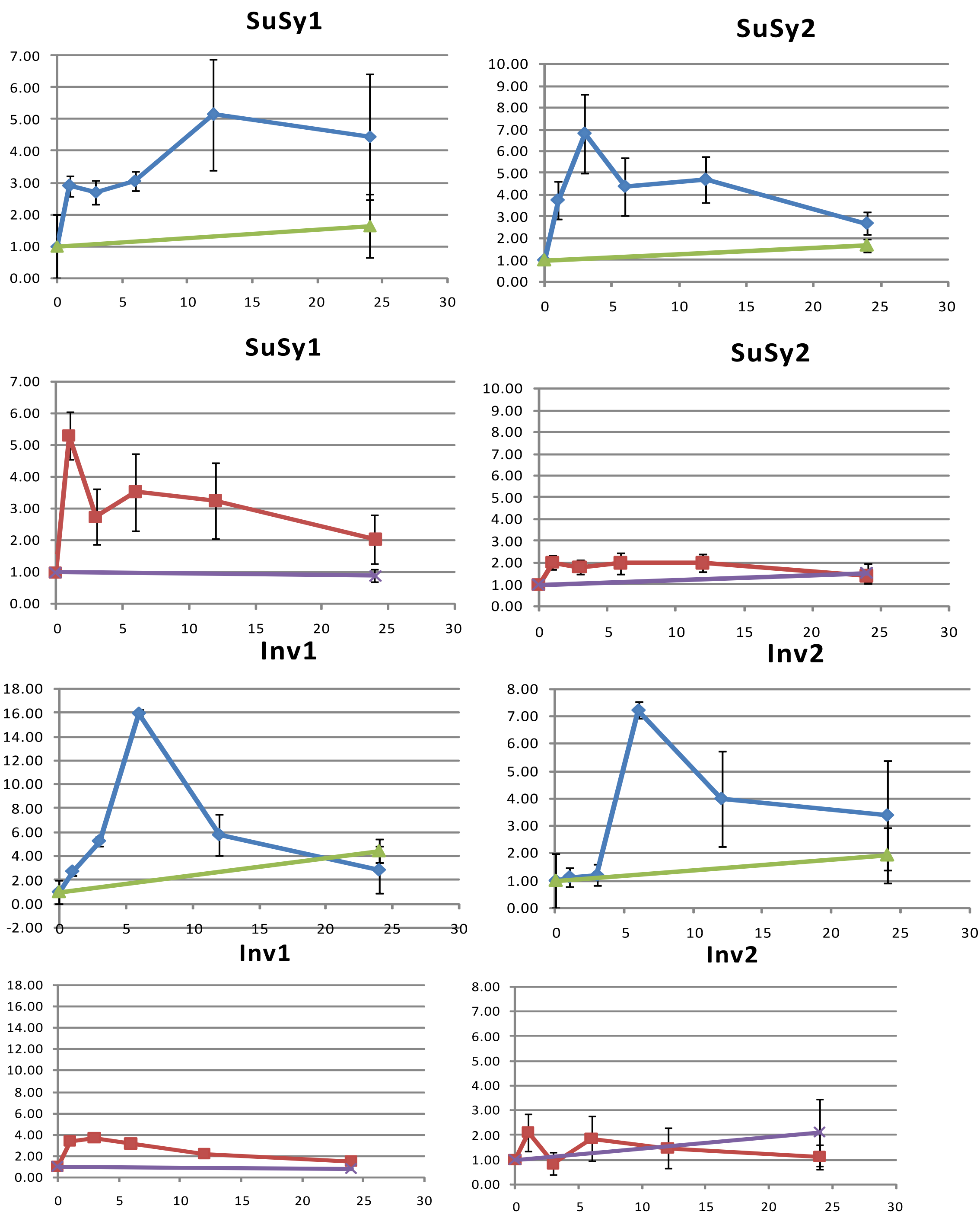


Fig 5. Real-time RT-PCR of PDC, SUS and INV gene expressions. Three-day-old seedlings were submerged for 1, 3, 6, 12 and 24 hours; expression pattern of *PDC1*, *PDC2*, *PDC3*, *PDC4, SUS1, SUS2, INV1, INV2* were examined. Relative transcription level is shown (1 = expression of seedlings from the control group); bars are of standard error of mean.

anaerobic metabolism is regulated by the activities of pH-sensitive enzymes. Within this model, pyruvate produced by glycolysis is converted to lactate in a reaction catalyzed by LDH, an enzyme with an optimum at physiological pH. As the cytosol acidifies, LDH is progressively inhibited and PDC is activated because its pH optimum is lower than normal cytoplasmic pH.

Within these experiments, three-day-old seedlings were completely submerged for 1, 3, 6, 12 and 24 hours then root samples were collected to examine the expression pattern of ADH and PDC. In ADH1 and ADH2



expressions, FR13A and IR64 have interestingly adverse expression amounts between the isozymes; however, they all peak in the first hour of submergence. It is still unclear whether the adversely different expressions of the ADH1 and ADH2 genes within the two cultivars have what kind of significance. The two ADHs have extremely similar sequences and are known to have different promoters. Native-PAGE was conducted in hope to be able to differetiate the two isozymes but in vain. What was observed in return was that ADH had stronger and earlier activity in FR13A under submergence stress, which enhances the proposal that ethanol fermentation is essential to a plant's ability to tolerate conditions of O_2 deficiency. In other studies, progenies of crossed plants overproducing both PDC and ADH showed individual anoxia tolerance; but increase of individual genes either had no effect on

or reduced the anoxia tolerance (Rahman et al., 2001).

Also asscociated in the ethanol fermentation pathway is pyruvate decarboxylase. There is also a different preference of the cultivars towards the isozymes. FR13A induces PDC4 much more prominently as IR64 specificly induces PDC1. The sequences of the spliced forms of PDC1 and PDC4 are extremely similar but known also to have different promoters (unpubished data). Thus the differential regulation of isozymes within the two cultivars may be due to different up-stream regulations. Other isozyme transcriptions concerned, it may be observed that IR64 has lower transcription levels of PDCs other than PDC1 when compared to FR13A.

SUS and INV genes were also examined, concerning the upstream of glycolysis, with the same treatments as the samples treated for ADH and PDC samples. There are two pathways for sucrose to link to glycolysis. The reversible plant-specific sucrose synthase (SUS) pathway that breaks sucrose into fructose and UDP-glucose requires hydrolysis of one molecule each of ATP and UDP per molecule of sucrose, whereas the unidirectional invertase pathway which breaks sucrose into glucose and fructose consumes two molecules of ATP per molecule of sucrose. The sucrose synthase pathway had been observed to be up-regulated and the invertase pathway repressed under hypoxia, but as the experiments show, thoush SUS is upregulated within both cultivars while FR13A has higher transcription folds, invertase within FR13A is observed to be also up-regulated under hypoxia. This is yet another cutting edge discover that has never been observed and controverses known proposed strategies of plant responses towards hypoxia. It is possible that the stress was not viewed as a threat by FR13A so much as to cut the ATP

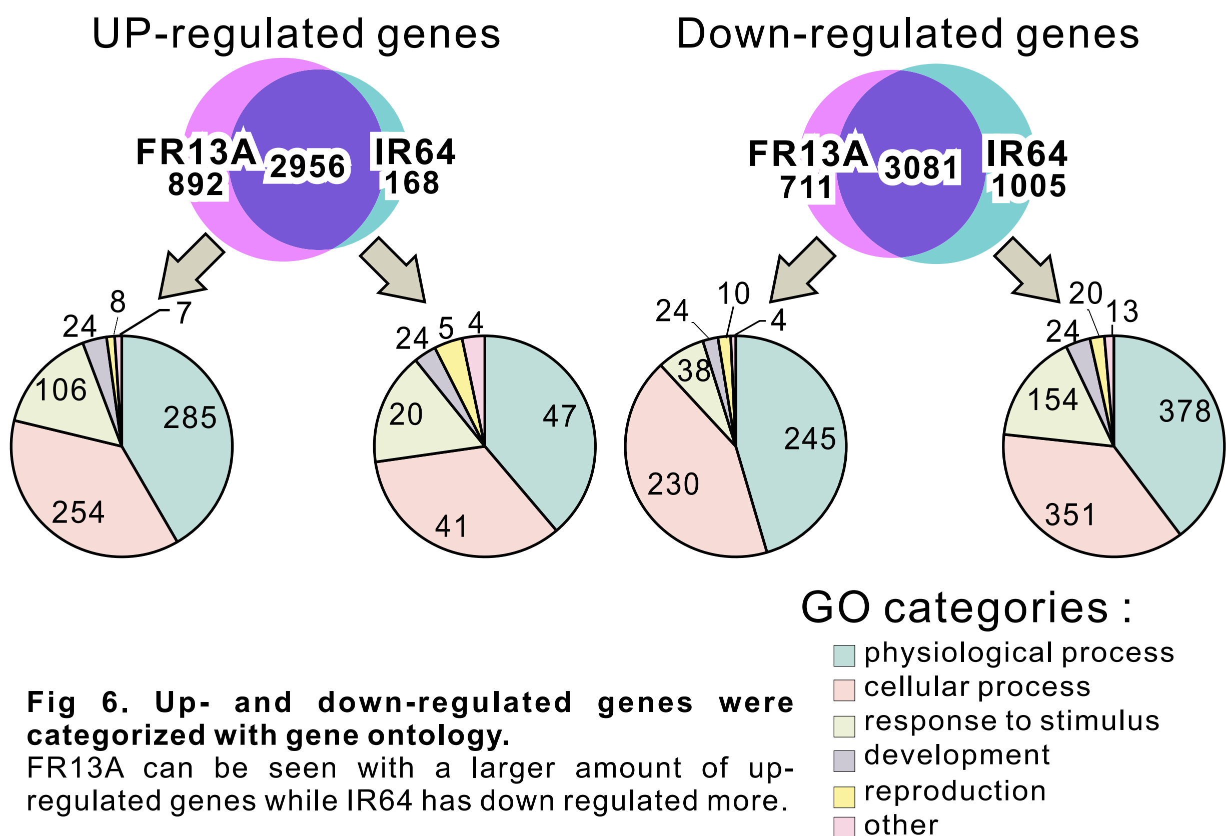


Fig 6. Up- and down-regulated genes were categorized with gene ontology.
FR13A can be seen with a larger amount of up-regulated genes while IR64 has down regulated more.

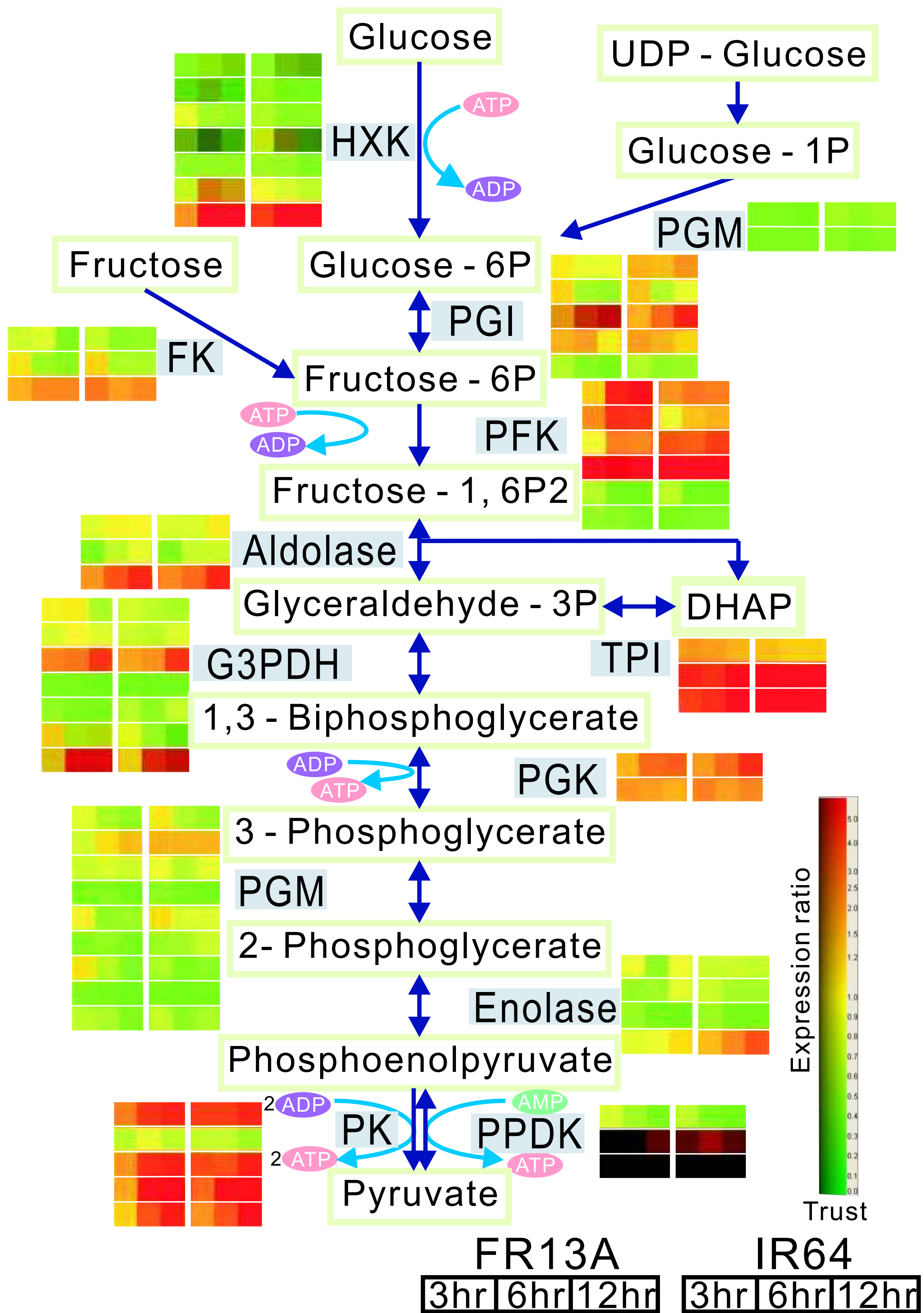


Fig 7. Effects of hypoxia on genes involved in Glycolysis.
Genes with up- or down-regulation within glycolysis and associated pathways are articulated on this figure. Relative expression levels of genes are indicated with color bars, scaled as the color ruler.

consuption of invertase, or that FR13A focuses more on increasing precursors of glycolysis to accelerate ATP generation so both SUS and INV are induced.

Microarray analysis

To determine gene expression profiles at genome wide scale, I obtained microarray data from Ms. Sandy Chen from Dr. Shih's lab. I compare the numbers of genes that are up or down regulated by more than 2 fold in IR64 and FR13A that were under submergence for 1, 3, 6, and 12 hours. It can be observed that FR13A has up regulated notably more genes than IR64, while IR64 has down regulated more genes than FR13A. This raises the possibility that FR13A has a relatively active molecular response to survive under submergence.

Through examination of all genes with gene ontology classification, genes participating in the physiologial processes can be seen with the largest scope of both up- and down-regulation, either by comparison with other gene ontology categories or with comparing the difference between the genes regulated in the two cultivars, followed by genes within cellular process and responsive genes towards stimulus.

This is consistent with the idea that rice plants need to make major physiological adjustment to cope with hypoxia stress.

Genes concerning glycolsis were investigated with the microarray data. It is said that the amount of stored carbohydrates is positively correlated with the level of submergence tolerance (Jackson and Ram, 2003), but then, PDC and ADH activities are not positively correlated with the depletion of carbonhydrate reserves or ethanol producing during submergence(Fukao et al., 2006). Thus the pathway of the ATP gaining process that occurs before fermentation, the glycolysis pathway was examined.

Within the microarray analysis, partial genes of glycolysis are down-regulated. This is fairly interesting, since most glycolytic genes were induced in maize and Arabidopsis, which are far more sensitive to flooding. Glycolytic gene regulation under submergence may therefore play an more important role within the overall metabolism, thus further research needed for clarifications of the unexpected mechanisms of glycolysis during hypoxia.

Abbreviations as followed:
HXK, hexokinase; PGM, Phosphoglucomutase; PGI, Phosphoglucoisomerase/ Glucose-6-phosphate isomerase; FK, fructokinase; PFK, phosphofructokinase; Aldolase, Fructose bisphosphate aldolase; TPI, Triosephosphate isomerase; G3PDH, NADP-dependent Glyceraldehyde-3-phosphate dehydrogenase; PGK, Phosphoglycerate kinase; PGM, Phosphoglycerate mutase; PK, pyruvate kinase; PPDK, pyruvate Pi dikinase.

Conclusions

FR13A adopted a combination of different strategies to cope with hypoxia stress caused by flooding, including reduced coleoptile growth, sustained aerenchyma formation, up-regulation of fermentative and sugar degrading genes, including ADH1, PDC4, SUS and INV, and higher ADH enzyme activity.

Through Gene Ontology classification, large scopes of gene expression difference between FR13A and IR64 were observed. Glycolytic genes, which have rarely been studied in rice, have been observed to be partially down-regulated under submergence. My findings point to a new direction of examining the regulation of glycolytical genes during submergence may help to elucidate the molecular mechanism responsible for flooding tolerance in different crop plants.

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