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Alteration of Protein Synthesis in Flooded Wax Apple (*Syzygium samarangense* Merr. et Perry) Roots

Änderungen in der Proteinsynthese gefluteter Wurzeln des Wachsapfels (*Syzygium samarangense* Merr. et Perry)

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Summary

Plantlets of wax apple (*Syzygium samarangense* Merr. et Perry), propagated from cuttings, were used to study changes of protein synthesis in roots under different flooding durations. Root tips of plantlets were labeled with ^{35}S-methionine, and the polypeptide patterns were analyzed using two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography. The labeling patterns of non-flooded controls and specimens of different flood durations (12, 24, 72 and 120 hours of flooding) were similar. Approximately 200–300 polypeptides could be discerned. However, there were differences in individual polypeptides among the various flood treatments. Compared to the polypeptide profile of non-flooded controls, there were 15, 20, 12 and 16 enhanced polypeptides, and 5, 9, 11 and 5 repressed polypeptides in 12, 24, 72 and 120 hours of flood treatments, respectively. Changes in polypeptides among the treatments were classified into five groups. Group 1 proteins were those that were enhanced or repressed only at certain flooding durations. Group 2 proteins were those that remained enhanced or repressed for 12 or 48 hours. Group 3 proteins were those that were unique to flooding stress. Group 4 proteins were those that remained constant under aerobiosis and flooding stress, and group 5 proteins were those that were unique to the aerobic environment.

Zusammenfassung

Durch Stecklinge vermehrte Pflanzen des Wachsapfels (*Syzygium samarangense* Merr. et Perry) wurden verwendet, um die Proteinsynthese in den Wurzeln in Abhängigkeit von der Überflutungsdauer zu untersuchen. Die Wurzelspitzen wurden mit ^{35}S-Methionin markiert und das Polypeptidmuster mittels zweidimensionaler Gelelektrophorese (SDS-PAGE) und Fluoreszenz analysiert. Die Markierungsmuster von nichtgefluteten Kontrollen und Proben mit unterschiedlicher Flutungsdauer (12, 24, 72 und 120 Stunden) sind ähnlich. 200 bis 300 Polypeptide konnten unterschieden werden. Allerdings gab es bei einzelnen Polypeptiden Unterschiede zwischen den einzelnen Überflutungs-Behandlungen. Im Vergleich zum Polypeptid-Profil der nichtgefluteten Kontrollen, gab es bei den Proben mit 12, 24, 72 und 120 Stunden Flutungsdauer 15, 20, 12 und 16 zusätzliche beziehungsweise 5, 9, 11 und 5 unterdrückte Polypeptide. Die Unterschiede zwischen den Polypeptiden der Behandlungen wurden in fünf Gruppen eingeteilt. Als Proteine der Gruppe 1 wurden diejenigen zusammengefaßt, die nur bei einer bestimmten Flutungsdauer ausgeprägt oder unterdrückt wurden. Gruppe 2 Proteine sind diejenigen, die bei 12 oder 48 Stunden ausgeprägt oder unterdrückt werden. Gruppe 3 Proteine sind bezeichnend für Überflutungsstress. Gruppe 4 Proteine blieben unter aeroben und anaeroben Bedingungen konstant, und Gruppe 5 Proteine sind kennzeichnend für aerobe Bedingungen.

Introduction

The expression of plant genes can be influenced by genetic control in response to environmental stress such as high temperature, drought and flooding (ADAMS and RINNE 1982, SACHS and HO 1986). The changes in gene expression that result from anaerobic stress are regulated at both transcriptional and posttranscriptional levels (BAILEY-SERRES and FREELING 1990, GERLACH et al. 1986, HAKE et al. 1985, HO and SACHS 1989, HOWARD et al. 1987, KENNEDY et al. 1992, OKIMOTO et al. 1980). Several genes involved in the anaerobic response have been isolated and characterized (SACHS 1994, SACHS et al. 1996). The influence of anaerobic stress on protein synthesis varies with different species. The synthesis of aerobic proteins in maize roots is inhibited under anaerobic stress and a new group of proteins is synthesized (SACHS and FREELING 1978). Of these new proteins, twenty have been identified as anaerobic polypeptides, ANPs (SACHS et al. 1980), or anaerobic stress proteins, ASPs (NOVER 1989). Most of these maize root anaerobic proteins were found to be enzymes of glycolysis or sugar-phosphate metabolism (SACHS et al. 1996). However, in rice embryos and shoots, there are many proteins, including aerobic proteins, that can be synthesized under anaerobic stress (MOCQUOT et al. 1981, MUJER et al. 1993). Most plants used to study protein synthesis and gene expression under flooding or anaerobic stress are herbacious monocots, such as maize (OKIMOTO et al. 1980, SACHS et al. 1980, 1996) and rice (MOCQUOT et al. 1981, MUJER et al. 1993), or dicotyledons, such as

soybeans (RUSSELL et al. 1990). However, the study of woody plants is quite limited.

The wax apple, a perennial woody fruiting plant, is grown in subtropical Taiwan as one of the top cash crops. Its normal fruiting period in Taiwan is from May to July. However, as a result of various treatments, including long term flooding, root pruning, girdling, growth retardant application and canopy pruning (SHU et al. 1990), the fruiting period has been advanced to the previous December. Among these treatments, flooding was one of the earliest methods that was commonly used. 45 days of constant flooding during the summer not only brought forward the wax apple harvest period by five months, but also increased harvest intervals and the productivity (WANG 1983). The physiological response and adaptation of wax apple trees to flooding stress is well documented (HSU et al. 1997, 1999, LIN and LIN 1992). Under flooding stress, nitrogen metabolism is inhibited, ADH activity increased and three new isozymes are induced in wax apple roots (HSU et al. 1997, LIN and LIN 1992). These studies indicate that protein synthesis in wax apple roots is influenced by flooding stress. In this report, we mimicked the field flooding conditions used to force wax apple trees to examine the changes in protein synthesis patterns in wax apple roots under different flooding durations.

Materials and Methods

Plant materials and flood treatments

Plantlets of wax apple (*Syzygium samarangense* Merr. et Perry) were propagated from two node long cuttings. The cuttings were collected from the most recent mature growth flushes of 3 year old container grown wax apple trees, and rooted in plastic net baskets (35 cm x 28 cm x 10 cm) with peat and bark compost (1:1, v/v). The rooting cuttings were cultivated in a net-house without temperature control and were watered by an automatic spraying system for six months.

Plantlets about 18–20 cm in height, with 3 pairs of leaves, and 10–15 branched adventitous roots, were exposed to five treatments: non flooded (control), and flooded for 12, 24, 72 and 120 hours. For the flooded treatments, water levels were maintained about 5 cm above the medium surface, as per field flooding practices. At the end of the flooding treatments, no obvious change in appearance both in the shoots and roots was observed. Root tips about 5–6 cm in length were collected and used for in vivo labeling.

In Vivo labeling and extraction of soluble protein

Two grams of root tips from each treatment were washed under running water, rinsed with distilled water, and cut into 3 cm sections. An incubation buffer (0.01 mol/l HEPES, 20 mmol/l $CaCl_2$, pH 6.0) containing 300 µCi of ^{35}S-methionine (1,450 Ci/mmol, Amersham, UK) was prepared. The roots were placed in 6 ml incubation buffer, followed by vacuum infiltration for 15 minutes to facilitate ^{35}S-methionine adsorption. The roots were incubated at room temperature for 4 hours. At the end of the incubation, the samples were washed three times with distilled water before protein extraction.

Protein was extracted according to SCHUSTER and DAVIES (1983) with slight modification. Samples were dry-crushed in a mortar cooled with liquid nitrogen. The tissue slurry was homogenized at 0 °C in 2 ml g^{-1} of extraction buffer (0.7 mol/l sucrose, 0.5 mol/l Tris, 30 mmol/l HCl, 50 mmol/l EDTA, 0.1 mol/l KCl, 2 % 2-mercaptoethanol, 5 % soluble PVP, 5 % PVPP). After centrifugation of the samples at 35,000 g for 30 minutes at 4 °C, the supernatant was filtered through Miracloth and centrifuged again (35,000 g, 30 minutes, 4 °C). The same volume of water saturated phenol was added to the supernatant and vortexed for 10 minutes. The mixture was then centrifuged at 35,000 g for 20 minutes at 4 °C. The same volume of extraction buffer (without PVPP) was added to the phenol phase, vortexed for 5–10 minutes and centrifuged again (35,000 g, 10 minutes, 4 °C). Three to five volumes of ice-cold methanol buffer (0.1 mol/l ammonium acetate and 10 mmol/l 2-mercaptoethanol in methanol) was added to the phenol phase and the proteins were allowed to precipitate at –20 °C overnight. After centrifugation at 5,000 g for 20 minutes at 4°C, the pellet was washed and vortexed with 1 ml ice-cold methanol buffer and transferred to a 1.5 ml eppendorf tube and centrifuged (5,000 g, 5 minutes, 4 °C). The pellet was washed with ice-cold methanol buffer and centrifuged again. The protein pellet was air-dried and resuspended in ice-cold methanol buffer and stored at –70 °C.

Two dimensional gel electrophoresis of protein

Two dimensional electrophoresis was performed according to O'FARREL (1975). The ampholytes in the isoelectric focusing (IEF) gels were four parts pH 5–7 ampholytes to one part pH 3.5–10 ampholytes. All SDS-polyacrylamide gels consisted of a linear gradient of 10 %–14 % (w/v) polyacrylamide. Samples containing approximately equal amounts of trichloroacetic acid (TCA)-precipitable radioactive protein were loaded onto the IEF gels and focused for 18 hours at 400 V, followed by 1 hour at 800 V. SDS gels were run at 20 mA per slab (constant current) for 4–5 hours until the dye reached the edge of the gel. Gels were stained with silver nitrate to reveal the molecular weight markers.

Fluorography

After destaining, gels were prepared for fluorography using the procedure of LASKEY and MILLS (1975). Dried gels were autoradiographied using Kodak X-OMAT XAR-5 film at –70 °C. Length of time for fluorography was determined by the amount of radioactivity applied on the first-dimensional gel, for a 10 day exposure of 1.0 x 10^6 disintegrations per minute (dpm).

Analysis of polypeptide profiles

Three replicates were prepared for each flooding duration. The resulting protein profiles were analyzed for differences between treatments. Non reproducible spots were ignored. All efforts were concentrated on detecting the presence or absence of spots for each treatment.

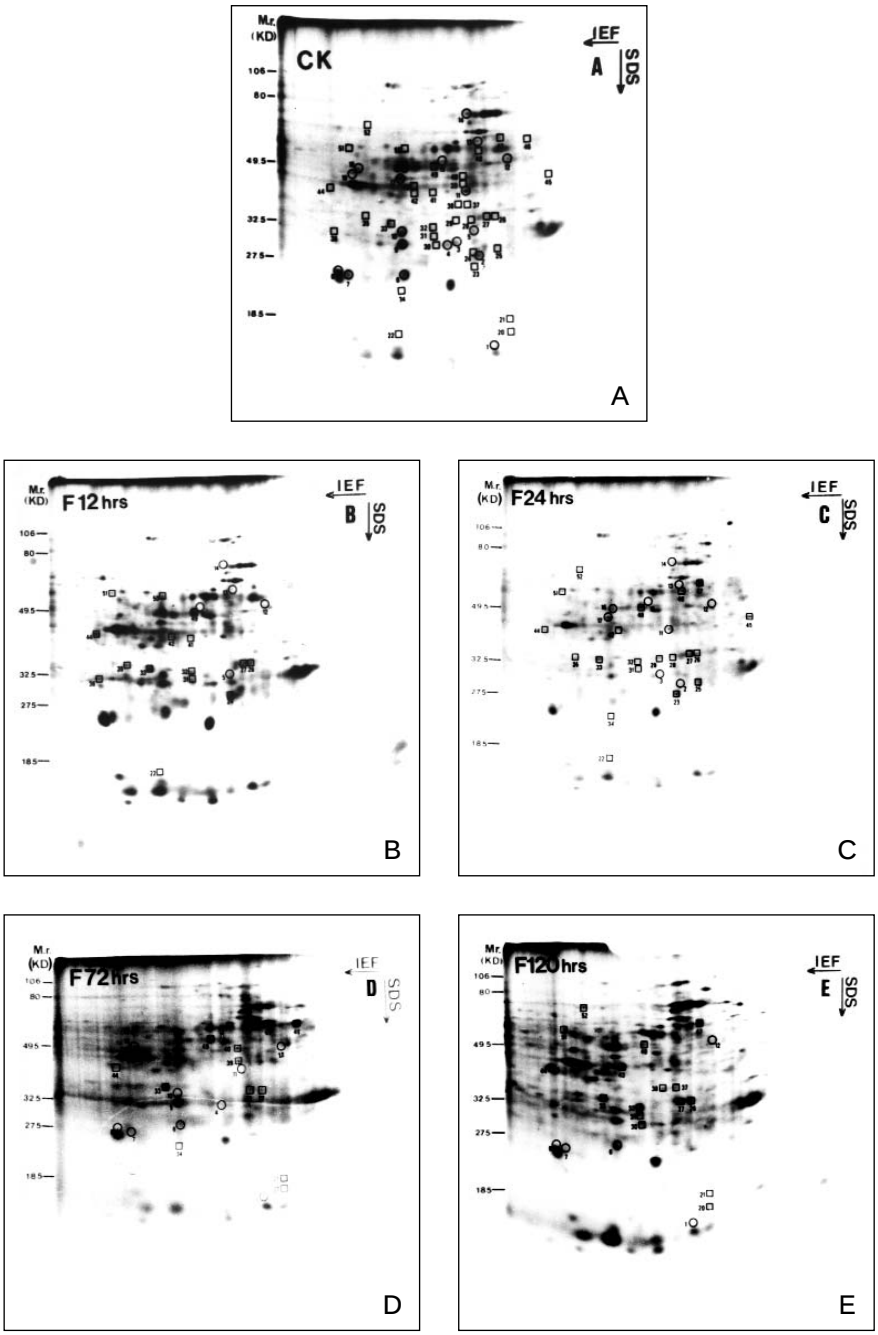


Fig. 1. Fluorographs of in vivo ^{35}S-methionine labeled polypeptides from different flood durations of wax apple fibrous roots resolved by two-dimensional gel electrophoresis. Plant cuttings were flooded for 0 (A), 12 (B), 24 (C), 72 (D) and 120 hours (E). The pI of the gels after IEF (first dimension) is 7.0 on the left and 4.5 on the right. SDS (second dimension) gels were run in 10% to 14% linear gradient polyacrylamide. Numbered squares indicate the increased or newly synthesized polypeptides in flooded roots compared with non-flooded controls. Numbered circles indicate the decreased polypeptides or polypeptides that disappeared during flooding.
Fluorographe von in vivo mit ^{35}S-Methionin markierten Polypeptiden aus unterschiedlich lang überfluteten Faserwurzeln des Wachsapfels, die mittels zweidimensionaler Gelelektrophorese aufgetrennt wurden. Die Stecklinge wurden 0 (A), 12 (B), 24 (C), 72 (D) oder 120 (E) Stunden geflutet. Der pI des Gels nach IEF (erste Dimension) beträgt links 7.0 und auf der rechten Seite 4.5. SDS (zweite Dimension)-Gele liefen in 10 bis 14% linear geneigten Polyacrylamiden. Nummerierte Quadrate kennzeichnen die in gefluteten Wurzeln im Vergleich zu nichtgefluteten Kontrollen verstärkt oder zusätzlich synthetisierten Polypeptide. Nummerierte Kreise bezeichnen Polypeptide, die beim Fluten weniger stark oder gar nicht gebildet werden.

Results

Soluble polypeptide changes in wax apple roots after 12, 24, 72 and 120 hours of flooding were examined. Fig. 1 shows the fluorographs of the two dimensional electrophorograms for each treatment. As shown in the figure, approximately 200–300 polypeptides could be resolved by two dimensional gel electrophoresis. In general, labeling patterns in controls and under different flooding durations were strikingly similar. However, quantitative and/or qualitative changes of the labeled spots did occur over the course of flooding. Table 1 summarizes the total changes in soluble polypeptides under the various flood durations.

After 12 hours of flooding, 15 increased or induced polypeptides were found in the flooded roots (polypeptide numbers 24, 36, 41, 50, 22, 35, 42, 33, 26, 27, 44, 49, 31, 32 and 51; Fig. 1B and Table 1). Among them, five polypeptides (numbers 33, 26, 27, 44 and 49) persisted through 120 hours of flooding. Three polypeptides (numbers 22, 35 and 42) were continuously synthesized for 24 hours of flooding. Three other polypeptides (numbers 31, 32 and 51) were continuously synthesized for 24 hours of flooding, but disappeared at 72 hours of flooding and were induced again after 120 hours of flooding.

Nine new polypeptides (numbers 23, 25, 28, 29, 45, 48, 34, 47 and 52) were induced after 24 hours of flooding (Fig. 1C and Table 1). Among these nine polypeptides, number 34 was continuously synthesized for 72 hours of flooding and number 47 remained throughout 72 and 120 hours of flooding. Number 52 was not de-

Table 1. Changes in protein synthesis of wax apple roots under different flooding durations
Änderungen in der Proteinsynthese von Wachsapfels-Wurzeln bei unterschiedlicher Überflutungsdauer

Polypeptide No.	Mr. (KDa)	Hours after flooding 12	24	72	120	Polypeptide No.	Mr. (KDa)	Hours after flooding 12	24	72	120
5[Z]	31	–[X]				24[Y]	28	+[W]			
13	58	–	–			36	31	+			
14	70	–	–			41	40.5	+			
15	49	–	–			50	55	+			
12	49	–	–	–	–	22	16	+	+		
2	27.5		–			35	32.5	+	+		
3	29		–			42	40.5	+	+		
16	47		–			33	32	+	+	+	+
17	43		–			26	32.5	+	+	+	+
11	41		–	–		27	32.5	+	+	+	+
4	28.5			–		44	41	+	+	+	+
9	29			–		49	47	+	+	+	+
10	31			–		31	30	+	+		+
18	47			–		32	31.5	+	+		+
19	44			–		51	55	+	+		+
1	15			–	–	23	26		+		
6	24.5			–	–	25	28		+		
7	24.5			–	–	28	32		+		
8	25			–	–	29	32		+		
						45	44		+		
						48	54		+		
						34	22		+	+	
						47	59		+	+	+
						52	64.5		+		+
						39	42			+	
						40	43.5			+	
						46	58			+	
						20	16.5			+	+
						21	18			+	+
						37	35				+
						30	28.5				+
						38	35				+
						43	42				+

[Z:] Numbered circles in Fig. 1. [X:] „–“- decreased or disappeared polypeptides during flooding. [Y:] Numbered squares in Fig. 1. [W:]“+“- increased or newly synthesized polypeptides during flooding.

tected at 72 hours of flooding, but was detected again after 120 hours of flooding.

At 72 hours of flooding, in addition to the seven polypeptides (numbers 33, 26, 27, 44, 49, 34, and 47) previously induced during flooding, five new polypeptides (numbers 39, 40, 46, 20 and 21) were induced (Fig. 1D and Table 1). Among these five polypeptides, numbers 20 and 21 were continuously synthesized for 120 hours of flooding. In addition to the polypeptides that were previously induced (numbers 33, 26, 27, 44, 49, 31, 32, 51, 47, 52, 20 and 21), an additional four polypeptides (numbers 37, 30, 38, and 43) were induced after 120 hours of flooding (Fig. 1E and Table 1).

When compared with non flooded controls, five polypeptides (numbers 5, 13, 14, 15 and 12) were depressed after 12 hours of flooding (Fig. 1B, Table 1). Among these, polypeptide 12 was suppressed throughout 24, 72 and 120 hours of flooding. Three polypeptides (numbers 13, 14, and 15) remained depressed after 24 hours of flooding. Five other polypeptides (numbers 2, 3, 16, 17 and 11) were repressed after 24 hours of flooding (Fig. 1C, Table 1). In addition to polypeptides 11 and 12, seven polypeptides (numbers 4, 9, 10, 18, 19, 1, 6, 7 and 8) decreased or were non-existent after 72 hours of flooding (Fig. 1D, Table 1). Among these, polypeptides 1, 6, 7 and 8 were continuously depressed for the full 120 hours of flooding (Fig. 1E, Table 1).

Discussion

The tolerance levels of plants to flooding or anaerobic stress vary between species. Thus, physiological response or gene expression is different among certain

species under flooding stress. The patterns of protein synthesis in non-flooded controls and during 12, 24, 72 and 120 hours of flooding are quite similar (Fig 1). Approximately 200–300 polypeptides were resolved by two-dimensional gel electrophoresis. These results are significantly different from other studies. Flood-stressed soybeans synthesized only four major proteins during eight hours of anaerobic treatment (RUSSELL et al. 1990). Maize (*Zea mays* L.) studies report that 20 polypeptides were synthesized during 5 hours of anaerobic treatment (OKIMOTO et al. 1980, SACHS et al. 1980). Our data are similar to four flood-tolerant *Echinochloa* species (MUJER et al. 1993) and rice (*Oryza sativa* L.) (MOCQUOT et al. 1981, MUJER et al. 1993, PRADET et al. 1985), which synthesized aerobic proteins under anaerobic conditions.

According to KELLY and FREELING (1982), gene expression and protein patterns in maize primary roots were different under low oxygen concentration (2% O_2), anaerobic (0% O_2) stress or aerobic (20% O_2) environment. In this study, the experiment was designed to mimic the farmer's field practices. No nitrogen or argon was pumped into the water during flooding, thus it might not have been strictly anaerobic. In addition, there are reports that aerenchyma formation occurs after a few days of flooding (DREW et al. 1979, KAWASE 1981); there is a direct relationship between aerenchyma formation and plant tolerance to flooding (DREW et al. 1985, JUSTIN and ARMSTRONG 1987). For wax apple, both control plants and 9-day-flooding-treated plants exhibited aerenchyma formation in the cortex tissue beginning 5 cm from the root tip (LIN and LIN 1992). The aerenchyma may help oxygen transportation from the above-water portion of the plant to the roots during flood stress (KAWASE 1981). Aerenchyma formation might explain why so many polypeptides were synthesized in our study.

Aerobic protein synthesis is a reversible reaction (KELLEY and FREELING 1982). The mRNA in aerobic proteins exist in the soybean root under anaerobic stress, but their translation is inhibited (RUSSELL et al. 1990). LIN and KEY (1967) reported that polyribosomes in soybean roots rapidly dissociated under anaerobic stress, but they could be returned to about 75% of the original level following restoration of aerobic conditions within 30 minutes. In our study, it took about one hour to clean, sample and segment the wax apple roots after different flood treatments. It is difficult to determine whether there was any inhibited mRNA reacting in the wax apple roots, which would make interpretation of the protein fluorographs more complicated. Furthermore, wax apple is a perennial woody plant, and its root structure is quite different from maize or soybean. Thus, the differences in protein fluorographs between soybean, maize and wax apple might be caused simply by the differences in plant characteristics.

In this study, the polypeptides can be categorized to five groups. Group 1 polypeptides are enhanced or repressed only at specific flood durations. There were newly enhanced or repressed polypeptides at each flood duration (Table 1). Group 2 polypeptides remain enhanced or repressed for 12- or 48-hour periods then disappear or are re-synthesized. Group 3 polypeptides are unique to flood stress (numbers 33, 26, 27, 44 and 49). Group 4 consists of constitutive polypeptides, which remained constant under flooding or normal treatment; most proteins in Fig. 1 belonged to this group. Group 5 polypeptides are unique to the aerobic environment, and are inhibited under flood stress (polypeptide 12).

Little information about these newly synthesized polypeptides currently exists. MUJER et al. (1993) suggested that for extended survival under anaerobic stress, most of the aerobic proteins must also be synthesized. The group 3 polypeptides (33, 26, 27, 44 and 49) that were unique to flooding might be similar to the anaerobic stress proteins of maize (SACHS et al. 1980). These polypeptides might have a close connection to the flood tolerance of wax apple roots.

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