¹H-NMR Imaging Analysis of Changes of Free Water Distribution in Barley and Soybean Seeds during Maturation**

Hiromi KANO, Nobuaki ISHIDA*, Toshio KOBAYASHI* and Mika KOIZUMI

(National Institute of Agrobiological Resources, Tsukuba Science City, Ibaraki 305 ;

* National Food Research Institute, Tsukuba Science City, Ibaraki 305, Japan)

Received October 30, 1989

Abstract : Free water content of barley seeds, as detected by a NMR, paralleled respiratory activity. Therefore, the amount and the mobility of free water were considered to be usable as indices of biological activity condition in seeds. The changes of the distribution map and the relaxation time of free water in barley and soybean seeds during maturation were traced by ¹H-NMR imaging (MRI). The disappearance of free water signal which is considered to indicate the decline of biological activities and the accumulation of stored materials such as polysaccharides and proteins, proceeded ununiformly in the endosperm of barley seed. Vascular bundles, each containing a water filled cavity, became thick at 14th day after heading, early stage of accumulation of stored materials, and it became thin following ripening, and no free water was detected in the vascular bundle at 39th day after heading. On the other hand, free water was detected uniformly in the developing seeds but not in those after the stage of maximum seed size and the dry seeds of soybean. The spin-lattice relaxation time (T_1) of free water in seed shortened while that in pod lengthened with the progress of ripening of soybean. Free water in rachises and awns was observed in barley after the late maturation stage and that in pod was also observed in soybean at the stage of maximum seed size. The proton signal due to triglyceride fatty acids stored in spherosomes was detectable in the dry soybean seed.

Key words : Barley, Free water, ¹H-NMR imaging, Seed maturation, Soybean.

¹H-NMR イメージングによるオオムギおよびダイズにおける登熟に伴う子実内自由水分分布の変化の解析 :
狩野広美・石田信昭*・小林登史夫*・小泉美香 (農業生物資源研究所・*食品総合研究所)

要 旨 : オオムギ子実における自由水 (NMR で観測し得る水) 含量と呼吸活性の間には強い相関が認められるので水の存在量と運動性を生物活性の指標として用いることができると考えられる。そこで、¹H-NMR イメージング (MRI) により登熟中のオオムギとダイズの子実における自由水の分布地図の変化を追跡した。オオムギにおいては、自由水の消失は胚乳で不均一に起こり、これは生物活性の低下とデンプンやタンパク質などの貯蔵物質の蓄積を示すと考えられる。オオムギでは出穂後 14 日目には維管束は太く見えるが登熟が進むとともに細くなり、39 日目には維管束の水が認められなくなった。一方、ダイズでは、肥大中の子実には自由水が均一に認められるが、最大肥大期以降の子実および乾燥子実では水のシグナルが認められず、子実の水は大部分貯蔵物質に結合して束縛された状態であると考えられた。子実中に自由水が認められなくなった登熟後期のオオムギの穂においても穂軸と芒には自由水が認められた。また、子実中には自由水が認められなくなった完熟期のダイズでも莢には自由水が認められた。乾燥ダイズでは弱い ¹H-NMR のシグナルが観測されたが、これは貯蔵脂肪に由来するものであると考えられる。

キーワード : ¹H-NMR イメージング, オオムギ, 自由水, ダイズ, 登熟。

Seeds of cereal crops accumulate and store materials of mainly polysaccharides and proteins accompanied with the decrease of water content and its mobility during maturation. Such a change of the physical status of water is considered to regulate the biological activities of tissues, because many enzymatic, metabolic and energetic processes occur in the water phase in the cells. Therefore, the obser-

vation of water is expected to provide useful information of physiological status in developing seeds⁶⁾.

¹H-NMR is a useful technique for analyzing water conditions *in vivo*^{6,11,13,19,21)}. Especially a magnetic resonance imaging (MRI) is attractive. It produces the maps of free water distribution in living tissues by using a static gradient and a radio-frequency magnetic field^{15,16,17)}. MRI has been developed as a means for medical diagnosis and recently begun to be applied for plant materials because of its usefulness in respect of morphological aspect. Omasa et al.²⁰⁾ imaged roots of *Vicia faba* seedlings and showed changes in

** A part of this investigation was presented at 186th meeting and 187th meeting of the Crop Science Society of Japan. The present work was supported in part by a Grant-in-Aid from the Ministry of Agriculture, Forestry and Fisheries (Bio Media Program, BMP89-VI 2).

water content of the seedlings in relation to water content in the soil where they were planted. Bottomley et al.²⁾ traced the uptake and movement of water in *Vicia faba* planted in soil medium using a paramagnetic NMR contrast reagent. Johnson et al.¹²⁾ investigated the difference in water content and the degree of binding in various stem tissues of *Pelargonium hortorum* between in the light and in the dark. Brown et al.³⁾ reported the water distribution map in *Pelargonium hortorum* root of less than 1 mm in diameter which corresponded to the pictures of root cross sections under an optical microscope. Ishida et al.⁹⁾ traced the changes of water distribution and its relaxation time in a tomato fruit during maturation and demonstrated that the different types of tissue in a fruit was distinguishable and that the change of the relaxation time of water indicated the physiological alternations of the tissues.

In this investigation, the changes of the distribution maps of free water in barley and soybean seeds during maturation were examined by ^1H -NMR imaging. The changes of relaxation time of water were also measured for soybean seed. And the process of seed maturations was discussed in relation to the biological and the morphological characteristics of the two crops.

Materials and Methods

Plants ; Barley (*Hordeum vulgare* L. cv. New Golden) was cultivated in soil culture using 1/2,000 a pots in the open field from October to June of the next year. Barley ears were harvested with the progress of growth, at 14th, 26th and 39th day after heading, and used as materials for measurements of ^1H -NMR images. Soybean (*Glycine max* Merr. cv. Enrei) was sown on 15th of April and cultivated in soil culture using 1/5,000 a pots in a green house. Plants began to flower at the beginning of June and the cultivation of the plants was finished at the beginning of August. During this period, pods containing seeds were harvested on 15th and 30th of June, 7th and from 13th to 26th of July for measurements of ^1H -NMR images. Relaxation time (T_1) values of water were calculated from image data.

Measurements of water distribution and its spin-lattice relaxation times (T_1) ; A NMR-spectrometer with a super conducting magnet operating

at 270 MHz (JEOL GSX-270) was used for obtaining ^1H -NMR images⁹⁾. An intact ear or a pod was fixed on a sample holder and set at the center of the 5.0 cm diameter detection coil of an imaging probe and inserted into a magnet which was equipped with another coil producing a gradient (15 mTesla/m) for the magnetic field of the apparatus. A spin-echo imaging technique was used and the echo time was 36 ms. A 256×256 array was applied to create a proton image with a resulting spatial resolution of $0.2 \text{ mm} \times 0.2 \text{ mm}$ and a 2.0 mm slice thickness (0.08 mm^3). Three imaging pulse sequences with different repetition times (0.5, 1.5 and 5.0 s) were used to measure the spin-lattice relaxation times (T_1) of the water by the progressive saturation method. The images were displayed on a color TV display (256×240 points). After the measurement of ^1H -NMR images, samples were cut according to the slice section and pictures of the section were taken.

Measurements of spin-spin relaxation times (T_2) and respiratory activities of barley seeds ; To control the water content, dry barley seeds were placed in a desiccator with saturated salt solution, the relative humidity of which was known⁷⁾. Then the desiccator was left at 5°C for one week to obtain the water content at equilibrium with its relative humidity. Intact barley seeds were placed in a 5 mm sample tube and spin-spin relaxation times (T_2) (the

Fig. 1. Relationship among free water, relaxation time (T_2), and O_2 uptake of barley seed in relation to water content. Water contents in seeds were adjusted strictly by equilibration with salt solutions with known humidity pressures. Symbol of (—●—) indicates free water content, (—△—) relaxation time (T_2) and (—○—) O_2 uptake.

Fig. 2. ^1H -NMR images of barley seed at different stages during maturation; vertical (a) and horizontal (b) sections at 14th day after heading; top (c), middle (d) and bottom (e) node order of the ear at 26th day after heading; vertical (f) and horizontal (g) sections at 39th day after heading. The horizontal lines shown by an arrow in the middle of imaging sections are the artifacts from the characteristics of the imaging apparatus.

Hahn method) was measured at room temperature (25°C) by high resolution NMR using a spectrometer above mentioned. Endogenous oxygen uptake of barley seeds was measured in a closed chamber by an oxygen electrode at 25°C ⁷⁾.

Results and Discussion

The physical status, expressed as the amount and the mobility of water, regulates biological activities in plant tissues^{6,7,8,10)}. This is because water phases are the primary field of biological processes such as enzymatic reactions, transportation and accumulation of

materials. The water detectable by a NMR is a sort of free water with mobility, which is restricted by interactions with solutes in plant cells. Macromolecular compounds severely prevent the mobility of water. Low molecular compounds restrict water mobility not strongly at low concentrations but the restriction grows strong with the increase of the concentration of the compounds. The relationship among the amount and the spin-spin relaxation time (T_2) of water detected by a NMR, and O_2 uptake activity in barley seeds is shown in Fig. 1, the water contents of which were adjusted strictly by equilibration with salt solutions with

known humidity pressures. The amount of water detectable by a NMR was negligibly small at 7% of water content and increased

Fig. 3. The changes of ^1H -NMR image in soybean seed during maturation in relation to the water content; the seed setting (a), the early (b), middle (c) and maximum seed size (d) stages of development and ripened dry seed (e). The arrow is the same as Fig. 2; indicates the artifact from the characteristics of the imaging apparatus.

along with increase of water content above 10%. The value of T_2 which indicates the mobility of water molecule, increased rapidly from 7% to 20% but not significantly above 20%. The water observed below 20% is considered to be loosely bound by stored materials of mainly macromolecular compounds such as polysaccharides and proteins in the seeds and that above 20% to be a free water. Oxygen uptake activity increased with the increase of the water detectable by a NMR and it paralleled the amount of free water above 20% of water content in seeds. From these findings, it seems reasonable to conclude that the places where free or loosely bound water presents, correspond to the biologically active parts of tissue and that the mobility of water correlates with the biological activity in the cells of tissue. Therefore, the distribution maps of water in barley seeds and the distribution maps and relaxation times (T_1) of water in soybean seeds were examined by a ^1H -NMR imaging to trace the changes of biological activity in relation to the morphogenesis during maturation.

Images of barley ears are shown in Fig. 2. On the 14th day after heading, a large amount of water in the seeds was observed along the sieve tube in the vascular bundle including nucellar projection and the glume of grain (Fig. 2a, b), which corresponds to the result obtained for wheat^{4,5}). Disappearance of water signal occurred heterogeneously in endosperm from middle parts between palea and vascular bundle with water filled cavity. The water in

Fig. 4. The change of signal intensity by the imaging pulse sequence with different repetition times (0.5, 1.5 and 5.0 s) in the seed and pod of soybean at the middle stage of seed growth.

Table 1. Changes of water content and relaxation time (T_1) during maturation of soybean.

Growth stage	Fresh weight of seed (mg)	Water content of seed (%)	T_1 (s)	
			Seed	Pod
(a) Seed setting stage	3	85.0	1.15	0.47
(b) Early stage of seed growth	54	85.0	0.64	0.58
(c) Middle stage of seed growth	383	71.0	0.72	1.00
(d) Stage of maximum seed size	683	62.4	0.56	1.20
(e) Seed drying stage	295	17.7	0.37*	—

The samples used for the measurements of water distribution maps in Fig. 3 were used. Symbol of (*) indicates the value of stored fatty acids.

Fig. 5. ^1H -NMR images with different relaxation times. The images were created from those acquired with different repetition times used for the measurement of relaxation times; long, (the image with 5 s repetition time) – (that with 1.5 s); medium, (the image with 1.5 s repetition time) – (that with 0.5 s); short, the image with 0.5 s repetition time. These images indicate the approximate amounts of water with T_1 values more than 1.5 s (long), from 0.5 to 1.5 s (medium) and less than 0.5 s (short). Top (a) shows the images at the middle stage and bottom (b) at the stage of maximum seed size.

the parts of tissue where the signal was not detected, was considered to be bound by stored materials which were accumulated in endosperm. Waters in glume, seed coat, pericarp and aleuron layer were not resolved by the apparatus used in this investigation. Water

remained in the glume, rachises and awns at 26th day after heading (Fig. 2c, d, e). The bridge of the endosperm grew in a radial direction and endosperm became thick, while vascular bundles grew thin at this stage. Grains ripened faster in the seeds at the top

node than at the bottom node of an ear. Water in seeds was no longer detected at 39th day after heading (Fig. 2f, g), while water remained in rachises and awns after ripening of the grains.

The changes of water distribution map, water content and its relaxation time in soybean during maturation are shown in Fig. 3 and Table 1. Large amounts of water were detected both in pod and seed before the stage of maximum seed size. The water signal in the seed became weak while water was observed in the pod at the stage of maximum seed size, when the water content in the seed was approximately 60%. Most of the water in the seed is considered to be bound by stored materials at this stage. Thenceforth the seed lost water and dried rapidly. The proton signal was detected in a dry seed; however, it is not attributed to water but fatty acids which were stored in the forms of triglycerides in spherosome⁶⁾.

The intensity of signal is reduced by imaging pulse sequence with short repetition times in the seed and pod of soybean at the middle stage as shown in Fig. 4. Using the image data with different repetition times, spin-lattice relaxation times (T_1) were calculated by the progressive saturation method, and the approximate amounts of water with different mobility (T_1) were calculated as shown in Fig. 5. Though the water contents in seeds were different between at the middle stage of seed growth and the stage of maximum seed size, the ratio of water amount with high mobility was low in the seeds at both stages. This is reflected apparently in the fact that T_1 values were as short as 0.7 and 0.6 s (Table 1). The T_1 of water indicates the mobility of the water molecules as well as T_2 . Usually the water in the physiologically active cells shows T_1 values longer than 1.0 s⁹⁾. The value of T_1 is shortened when stored materials such as polysaccharides and proteins are accumulated in the cells, associated with the retardation of the activity of biological reactions. Relaxation times (T_1) of water in the seed and the pod of soybean were changed contradictory (Table 1). The relaxation time in the seed, which is as long as 1.2 s at the seed setting stage, shortened with seed development, while its value in the pod increased with maturation till the stage of maximum seed size. Changes of T_1

values indicate that the seed cells showed the nature characteristics of a storage organ after the early stage of seed growth while the pod played a role as a temporary storage to pass photosynthates to the seed before the early stage of seed growth. And the capacity to accumulate materials in the seed as a sink increased with the development of the seed and photosynthates were translocated directly into the seed. On the other hand, the pod maintained high water mobility and biological activity to acquire high integrity of cells for protecting the seeds till the seeds begin drying.

The ripening process of seed was different between barley and soybean. The increase of the size of grain preceded the increase of the content in barley seed¹⁸⁾ and grains were ripened ununiformly in endosperm from middle parts between palea and vascular bundle (Fig. 2) and enlarged by accumulating the photosynthates transported through vascular bundle^{4,5)}. On the other hand, soybean seed accumulated materials with the increase of the size (Fig. 3, Table 1)^{1,14)}. The rachises and awns maintained the biological activity long after the water got undetectable in barley seed (Fig. 2), suggesting that the seeds are still maintaining organic relations with crop plant body and slowly accumulating photosynthates with the expense of the water remaining in them which is not detected by a NMR (Fig. 2). As for soybean, the seed and the pod lost water rapidly associated with the decrease of their size¹⁴⁾, indicating a rapid decrease of biological activity (Fig. 1) at the final stage of maturation (Fig. 3).

The distribution map and the relaxation time (T_1) of water distinguished among different types of tissues such as endosperm, vascular bundle, glume, rachis and awn in barley, and seed and pod in soybean. And the dynamic variations of biological activities are able to be traced without destruction of crop tissues by ¹H-NMR imaging.

References

1. Bills, R.F. and R.W. Howell 1963. Biochemical and cytological changes in developing soybean cotyledons. *Crop Sci.* 3 : 304—308.
2. Bottomley, P.A., H.H. Rogers and T.H. Foster 1986. NMR imaging shows water distribution and transport in plant root systems *in situ*. *Proc. Natl. Acad. Sci. USA* 83 : 87—89.

3. Brown, J.M., G.A. Johnson and P.J. Kramer 1986. *In vivo* magnetic resonance microscopy of changing water content in *Pelargonium hortorum* roots. *Plant Physiol.* 82 : 1158—1160.
4. Eccles, C.D., P.T. Callaghan and C.F. Jenner 1988. Measurement of the self-diffusion coefficient of water as a function of position in wheat grain using nuclear magnetic resonance imaging. *Biophys. Jour.* 53 : 77—81.
5. Hoshikawa, K. 1961. Studies on the ripening of wheat grain. 2. Development of the endosperm tissue. 3. Development of the starch grain and reserve protein particle in the endosperm. *Proc. Crop Sci. Soc. Japan* 29 : 415—420*.
6. Ishida, N., H. Kano, T. Kobayashi, H. Hamaguchi and T. Yoshida 1987. Estimation of biological activities by NMR in soybean seeds during maturation. *Agric. Biol. Chem.* 51 : 301—307.
7. ———, ———, ——— and T. Yoshida 1988. Analysis of physical states of water in soybean seeds by NMR. *Agric. Biol. Chem.* 52 : 2777—2781.
8. ———, T. Kobayashi and H. Kano 1988. Analysis of maturation process of soybean seeds by ¹H-NMR imaging. *Japan. Jour. Crop Sci.* 57 (Extra issue 2) : 235—236**.
9. ———, ———, M. Koizumi and H. Kano 1989. ¹H-NMR imaging of tomato fruits. *Agric. Biol. Chem.* 53 : 2363—2367.
10. ———, ———, ——— and ——— 1989. Analysis of water distribution changes during maturation of barley and sorghum seeds by ¹H-NMR imaging. *Japan. Jour. Crop Sci.* 58 (Extra issue 1) : 146—147**.
11. Iwaya-Inoue, M., K. Sakaguchi and S. Kaku 1989. Statistical studies using AIC method to decide the question of “break” or “straight” in Arrhenius plots of water proton NMR relaxation times in chilling-sensitive *Vigna* and insensitive *Pisum* seedling. *Plant Cell Physiol.* 30 : 309—316.
12. Johnson, G.A., J. Brown and P.J. Kramer 1987. Magnetic resonance microscopy of changes in water content in stems of transpiring plants. *Proc. Natl. Acad. Sci. USA* 84 : 2752—2755.
13. Kerhoas, C. and C. Dumas 1986. Nuclear magnetic resonance and pollen quality. In *Modern Methods of Plant Analysis Vol. 2, Nuclear Magnetic Resonance*. (Eds.) H.F. Linskens and J.F. Jackson, Springer-Verlag, Berlin, Heidelberg, New York, Tokyo. 169—190.
14. Konno, S. 1976. Physiological study on the mechanism of seed production of soybean. *Bull. Natl. Inst. Agr. Sci. D* 27 : 139—294*.
15. Lauterbur, P.C. 1973. Image formation by induced local interactions: Examples employing nuclear magnetic resonance. *Nature* 242 : 190—191.
16. ——— 1974. Magnetic resonance zeugmatography. *Pure Appl. Chem.* 40 : 149—157.
17. Mansfield, P. and I.L. Pykett 1978. Biological and medical imaging by NMR. *Jour. Magn. Res.* 29 : 355—373.
18. May, L.H. and M.S. Buttrose 1959. Physiology of cereal grain. II. Starch granule formation in the developing barley kernel. *Aust. Jour. Biol. Sci.* 12 : 146—159.
19. McCain, D.C. 1986. Orientation of chloroplasts in leaves by ¹H NMR spectroscopy. In *Modern Methods of Plant Analysis. Vol. 2, Nuclear Magnetic Resonance*. (Eds.) H.F. Linskens and J. F. Jackson, Springer-Verlag, Berlin, Heidelberg, New York, Tokyo. 127—147.
20. Omasa, K., M. Onoe and H. Yamada 1985. NMR imaging for measuring root system and soil water content. *Environ. Control Biol.* 23 : 99—102.
21. Wüthrich, K. 1976. *NMR in Biological Research : Peptides and Proteins*. North-Holland Publishing Company, Holland.

* In Japanese with English summary.

** In Japanese, the titles were translated by original authors.