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Genes with Different Modes of Inheritance Regulate Seed Germination in Preharvest-sprouting-tolerant Lines of Buckwheat (*Fagopyrum esculentum*)

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Abstract

Preharvest sprouting (PHS), caused by rain after maturity, leads to a severe degradation of buckwheat quality. We previously reported the PHS tolerance of several natural resources and landraces in Japan, and have bred PHS-tolerant lines. Here, we investigate the rates of PHS in our breeding lines and leading cultivars in Japan. The new lines 'Kyukei 28' and 'Kyukei 29' had better PHS tolerance than all leading cultivars. To reveal the PHS tolerance inherited by these lines, we performed genetic analysis using crosses between these lines and a self-compatible line, 'Kyukei SC7'. The rate of germination in F_2 segregating lines showed different segregation patterns, suggesting that major tolerance genes in 'Kyukei 28' (KY28) are recessive and those in 'Kyukei 29' (KY29) are dominant. This information could help to accelerate the breeding of buckwheat lines with PHS tolerance and develop DNA markers for the selection of tolerant lines.

Discipline: Crop Science
Additional key words: breeding, ecotype, genetic analysis, seed dormancy

Introduction

In most parts in Japan, buckwheat (*Fagopyrum esculentum* (L.) Moench)) can be cropped twice a year in summer and in autumn, although just once in autumn is more common. Summer cropping is becoming popular because it enables harvesting around June to supply fresh, high-quality buckwheat grain and flour to the Japanese market in summer, when the demand for buckwheat noodles soars (Shibata 1981, Vinning 2001).

The harvest time of buckwheat cultivars depends in part on ecotype. Buckwheat has four main ecotypes (i.e., summer, intermediate summer, intermediate autumn, autumn), depending on the response of the flowering time and ripening rate to the photoperiod (Nagatomo 1961, Lachmann & Adachi 1990). In summer cropping, summer and intermediate summer cultivars yield well, but the maturation of the other ecotypes is hindered by the long photoperiod, resulting in a poor yield (Hara et al. 2009a).

Preharvest sprouting (PHS) by which seeds germinate following rain after grain maturity (Fig. 1) is common in summer cropping because the rainy season starts around this time. PHS decreases the pasting viscosity and quality of buckwheat flour (Hara et al. 2007, 2009b). PHS in buckwheat has been reported not only in Japan but also in Korea and Australia (Choi et al. 1992, Bluett 2001, Morishita & Tetsuka 2001, Hara et al. 2007). It will be important to develop PHS-tolerant cultivars to ensure good flour quality.

PHS differs widely among buckwheat cultivars and landraces (Hara et al. 2008). We demonstrated that varietal differences in its occurrence in the field are closely correlated with differences in germination rate in the laboratory (Hara et al. 2008). Thus, germination testing is a useful method for evaluating the PHS tolerance of buckwheat cultivars. Using this method, we have developed PHS-tolerant cultivars 'Harunoibuki' (Hara et al. 2012) and 'NARO-FE-1,' which are suitable for summer cropping. These cultivars are now being grown in some parts of Japan and contributing to the stabilization of buckwheat quality. However, further improvement in PHS tolerance in buckwheat is necessary,

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Fig. 1. Preharvest sprouting of buckwheat

as higher air temperatures expected due to global warming raise the risk of PHS (Hara et al. 2008).

Genes or quantitative trait loci (QTLs) related to seed dormancy and PHS have been reported in *Arabidopsis* (Alonso-Blanco et al. 2003) and major crops such as wheat (Nakamura et al. 2011), rice (Sugimoto et al. 2010), and barley (Sato et al. 2016). In these species, both dominant and recessive genes or QTLs that control the level of seed dormancy have been reported (Buraas & Skinnes 1984, Sato et al. 2016).

To efficiently breed new cultivars with high levels of seed dormancy, gene pyramiding assisted by selection markers offers a promising strategy (Kumar et al. 2010, Tyagi et al. 2014). However, there is no information about such genes or QTLs in buckwheat. It is difficult to obtain genetic information by genetic analysis because buckwheat is a heteromorphic self-incompatible plant with two types of floral architecture: thrum (short style) and pin (long style). Thus, we developed several self-compatible (SC) lines with a long homostyle (LH) for use in the genetic analysis of agricultural traits and the identification of important genes (Matsui et al. 2007, 2008).

Here, we investigated the PHS tolerance of our breeding lines and cultivars, and found that two breeding lines—'Kyukei 28' and 'Kyukei 29'—have superior PHS tolerance. Genetic analysis showed that at least two different mechanisms regulate PHS tolerance.

Materials and methods

1. Plant materials

We evaluated the PHS tolerance of three breeding lines and seven cultivars grown in Japan (Table 1). 'Kyukei 28' (KY28) and 'Kyukei 29' (KY29) are lines bred through the mass selection of low-PHS individuals. KY28 was developed from 'Harunoibuki' (Hara et al. 2012) × 'Hitachiakisoba.' KY29 was developed from 'Kanoya-Zairai' × 'Hitachiakisoba.' 'Kyukei SC7' (KSC7) is a SC line produced from 'Asahimura-Zairai 3' × ('Hitachiakisoba' × 'Norin-PL1' [Matsui et al. 2008]). For PHS segregation testing, we developed F_2 populations from KY28-1 × KSC7 (cross A), KY28-2 × KSC7 (cross B), KY29-1 × KSC7 (cross C), and KY29-2 × KSC7 (cross D).

We also investigated the PHS tolerance of 30 breeding lines produced from two *F. homotropicum* lines—C9139 and C9255 (Matsui et al. 2003a). We investigated four BC_1F_6 lines produced from BTN × (BTN × C9139), and 20 F_6 and six F_9 lines produced from BTN × C9255 (Fig. 2).

2. Evaluation of preharvest sprouting tolerance of new lines and cultivars

Plants were grown at the National Agricultural Research Center for the Kyushu-Okinawa Region, NARO, Koshi, Kumamoto, Japan. Twenty plants from each plot were harvested after maturity and then threshed by hand for the evaluation of PHS tolerance.

PHS tolerance was evaluated as described in Hara et al. (2008). In brief, 50 seeds were immediately placed on filter paper saturated with distilled water in a Petri dish. The dishes were incubated in a germination cabinet at a constant 25°C in the dark. Dishes were checked once a day for seven days or longer, and then germinating seeds were counted and removed. The results are percentages.

3. Genetic analysis of preharvest sprouting

The F_2 populations of crosses A to D were grown in a field of the Institute of Crop Science, NARO, Tsukuba, Japan, in 2016 (crosses A, C, and D) and 2017 (cross B). The PHS tolerance of each plant was evaluated as described above with 20 seeds. Seeds on pin plants were obtained by open pollination. Before genetic analysis, we confirmed whether the populations could be used for genetic analysis by testing the segregation ratio of flower morphology, which is known to be controlled by one gene (*S*).

Results

1. Evaluation of preharvest sprouting in breeding lines and cultivars

Germination rates of the standard cultivars Kitawasesoba, Harunoibuki, Hitachiakisoba, and Kanoya-Zairai (Table 1) were similar to those previously reported (Hara et al. 2008). 'Kanoya-Zairai' with the lowest rate would apparently be a good parental line for breeding for

Table 1. Cultivars and breeding lines used in germination tests

Cultivar, breeding line	Origin or cross	Ecotype	Maturity date*	Germination rate (%)†	
				21 Oct.	29 Oct.
Standard cultivars used to confirm the level of preharvest sprouting					
Kitawasesoba	Botansoba	summer	18 Oct.	96	95
Harunoibuki	Hashikamiwase	intermediate summer	21 Oct.	73	89
Hitachiakisoba	Kanasago-Zairai	intermediate autumn	29 Oct.	51	72
Kanoya-Zairai	Landrace	autumn	14 Nov.	-	28
Cultivars and breeding lines					
Sachi-Izumi	Asahimura-Zairai 3 / Tsushima-Zairai	intermediate autumn	27 Oct.	-	86
Kyushu 2	Asahimura-Zairai 3 / Chushinkei VII	intermediate autumn	24 Oct.	-	70
Kyukei SC7 (KSC7)	Asahimura-Zairai-3' × ('Hitachiakisoba' × 'Norin-PL1')	intermediate autumn	29 Oct.	53	78
NARO-FE-1	Cross among Kitawasesoba, Yaita-Zairai, Asahimura-Zairai 3, Hashikamiwase, Hitachiakisoba, Chushinkei VII, Kyukei 30, and Kyukei 10	intermediate summer	21 Oct.	45	74
Kyukei 28 (KY28)	Harunoibuki × Hitachiakisoba	intermediate autumn	30 Oct.	-	20
Kyukei 29 (KY29)	Kanoya-Zairai × Hitachiakisoba	autumn	4 Nov.	-	17
Miyazakiwasekaori	Kanoya-Zairai	autumn	7 Nov.	57	77

*Maturity date was defined as the date when 80% of seeds on plants had blackened.
†Seeds were sown on 27 August, and 50 mature seeds were collected from each plant in the field on 21 and 29 October. Germination tests were performed immediately thereafter.
-: Not investigated

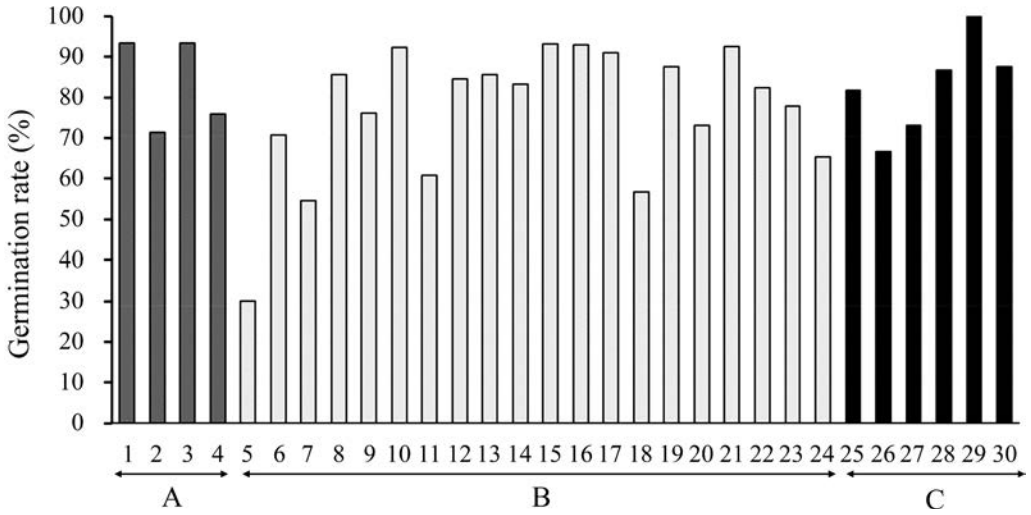

Fig. 2. Germination rates of progeny developed from self-compatible wild *F. homotropicum*
A: BC_1F_6 lines developed from *F. esculentum* 'Botansoba' (BTN) × (BTN × *F. homotropicum*)
B: F_6 lines developed from BTN × *F. homotropicum*
C: F_9 lines developed from BTN × *F. homotropicum*

PHS tolerance (Hara et al. 2008). The germination rates of seeds of all cultivars collected on 29 October were higher than those of seeds collected on 21 October (Table 1), except for 'Kitawasesoba,' whose germination rate was already as high as 96% on 21 October.

The germination rates of breeding lines 'KY28' and 'KY29' were lower than those of any other cultivars or lines, suggesting the promising PHS tolerance of these two lines. As both lines matured earlier than 'Kanoya-Zairai,' both would also be useful as parental lines to produce PHS-tolerant lines suitable for summer cropping.

We also investigated the germination rates of the progeny of crosses between *F. esculentum* and *F. homotropicum*. Given the strong seed dormancy of *F. homotropicum* (Wang & Campbell 2000), introducing this trait into the progeny would make it useful as a parental line. All four BC_1F_6 lines had high germination rates (71%-93%). All six F_9 lines also had relatively high germination rates (67%-100%). Moreover, the F_6 lines had a wide range of germination rates (30%-93%). All lines developed using

F. homotropicum have been selected without testing for PHS tolerance, resulting in the selection of plants with seeds that germinate easily (i.e., BC_1F_6 and F_9 lines). Some F_6 lines (5, 7, 11, and 18) had lower germination rates and may be useful as parental lines for breeding PHS-tolerant lines, although their shattering habit (i.e., brittle pedicels) must be removed (Matsui et al. 2003b).

2. Genetic analysis of two PHS-tolerant lines —'Kyukei 28' and 'Kyukei 29'

To clarify the inheritance of PHS tolerance in buckwheat, we performed genetic analysis with KY28 and KY29, which showed good PHS tolerance.

The segregation ratios of flower morphology in all populations were consistent with the expected ratio of 3:1 for a single dominant gene, thereby suggesting that the populations can be used for genetic analysis (Table 2).

We investigated the distributions of the frequency of PHS in the segregating populations derived from the four crosses (Fig. 3). Progeny derived from KY28 × KSC7 (crosses A and B) showed a broad distribution from 0% to 100%, averaging 78.9% in cross A and 58.6% in cross B.

Table 2. F_2 populations used for genetic analysis of PHS

Population	Cross combination*	Year	Number of plants	Segregation of flower morphology (LH:P)†	χ^2 value (3:1)	*P*
Cross A	KY28 (P)-1 / KSC7 (LH)	2016	133	100:30	0.256	$0.6<P<0.7$
Cross B	KY28 (P)-2 / KSC7 (LH)	2017	105	84:21	1.400	$0.2<P<0.3$
Cross C	KY29 (P)-1 / KSC7 (LH)	2016	109	88:19	2.994	$0.05<P<0.1$
Cross D	KY29 (P)-2 / KSC7 (LH)	2016	132	101:31	0.162	$0.6<P<0.7$

*Two different plants of KY28 and KY29 were used to produce segregating populations to reduce the possibility of genotypic differences related to PHS.
†Flower morphology could not be measured in three progeny of cross A and two of cross C.
P: pin; LH: long homostyle

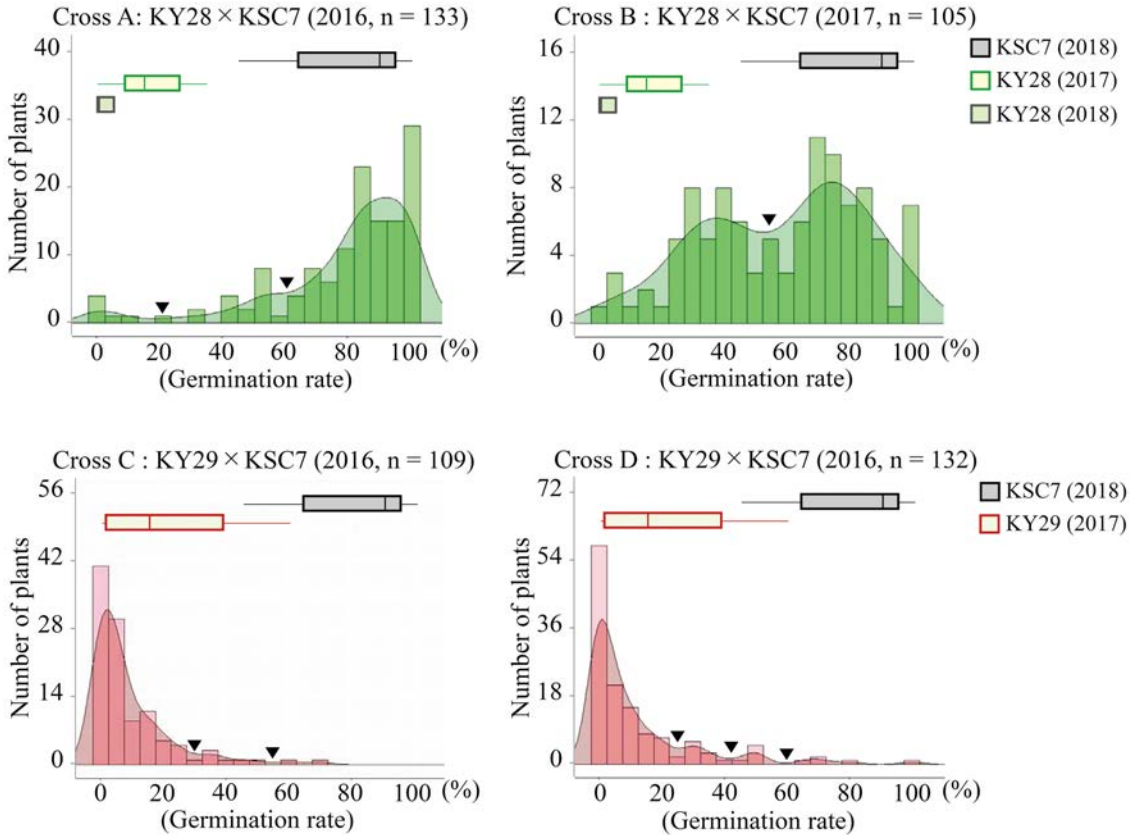

Fig. 3. Segregation of germination rate in the progeny of crosses A and B (KY28 × KSC7 in 2016 and 2017) and C and D (KY29 × KSC7 in 2016)
Curves represent kernel density estimates. Box plots show the germination rates of parents. Arrowheads indicate points of discrimination by χ^2 analysis (Table 3).

The apparent bimodal distributions suggest that major PHS tolerance genes in KY28 are recessive. However, progeny derived from KY29 × KSC7 (crosses C and D) showed high tolerance to PHS, averaging 9.2% in cross C and 11.9% in cross D. The biased distributions suggest that major tolerance genes in KY29 are dominant.

To deduce the number of PHS tolerance genes, we used the χ^2 test to test several expected ratios, using potential threshold values surmised from the inflection points determined by kernel density estimation (Table 3, Fig. 3). In the KY28 × KSC7 populations (crosses A and B), the segregation did not fit any expected ratio with one or two dominant genes (3:1 or 15:1), indicating that PHS tolerance was regulated by several recessive genes in those populations. Further study would be needed to develop selection markers. However, in both KY29 × KSC7 populations (crosses C and D), the segregation fit the expected ratio of two dominant genes (15:1), indicating that PHS tolerance was controlled by two major genes. However, the χ^2 and *P* values change with the threshold, so QTL analysis would be needed to explain the inheritance and develop selection markers.

Discussion

1. Estimation of the origin of resistance genes

KY28 and KY29 had the highest PHS tolerance (Table 1). KY28 was derived from 'Harunoibuki' × 'Hitachiakisoba.' 'Harunoibuki' could be a donor of tolerance genes in KY28, because it was developed as a PHS-tolerant cultivar for summer cropping by the selection of PHS-tolerant 'Hashikamiwase' plants (Hara et al. 2012), although 'Harunoibuki' showed lower PHS tolerance than KY28 in this test. (As buckwheat is allogamous, a single cultivar contains many heterozygous loci and some non-fixed traits, so selection is possible.) The difference in germination rate between KY28 and 'Harunoibuki' may be caused by different genetic backgrounds.

Cultivars with earlier maturity (including 'Harunoibuki') showed lower PHS tolerance in our results, although 'Harunoibuki' (Hara et al. 2008) and 'NARO-FE-1' are known for their improved tolerance in summer cropping. This contradiction can be explained by the breakage of seed dormancy by higher temperatures and the early maturity of these cultivars. Seed dormancy

Table 3. Observed and expected phenotypic ratios of preharvest sprouting in F_2 population

Population	Expected segregation ratio	Threshold (%)	Number of plants	χ^2 value	*P*
Cross A	3:1	20	5:128	360.00	$P<0.001$
		60	22:111	242.41	$P<0.001$
	15:1	20	5:128	1,838.21	$P<0.001$
		60	22:111	1,353.11	$P<0.001$
	9:7	20	5:128	148.91	$P<0.001$
		60	22:111	85.22	$P<0.001$
	12:3:1	20, 60	5:17:111	1,361.07	$P<0.001$
Cross B	3:1	55	48:57	48.03	$P<0.001$
	15:1	55	48:57	413.49	$P<0.001$
	9:7	55	48:57	4.74	$P<0.05$
Cross C	3:1	30	101:8	18.13	$P<0.001$
		55	107:2	31.20	$P<0.001$
	15:1	30	101:8	0.22	$0.6<P<0.7$
		55	107:2	3.63	$0.05<P<0.1$
	12:3:1	30, 55	101:6:2	18.13	$P<0.001$
Cross D	3:1	25	110:22	4.89	$P<0.05$
		40	121:11	19.56	$P<0.001$
		60	127:5	31.68	$P<0.001$
	15:1	25	110:22	24.44	$P<0.001$
		40	121:11	0.98	$0.3<P<0.4$
		60	127:5	1.37	$0.2<P<0.3$
	12:3:1	25, 60	110:11:11	9.78	$P<0.01$
	9:3:3:1	25, 40, 60	110:11:6:5	40.34	$P<0.001$

in buckwheat is strongly influenced by temperature (Hara et al. 2008, Samimy 1994, Wang & Campbell 2000). In autumn cropping, early maturing lines mature under still warm temperatures, so the seed dormancy of 'Harunoibuki' could be quickly broken by warm temperatures. In fact, the germination rates of seeds of all cultivars collected on 29 October were higher than those of seeds collected on 21 October (Table 1), indicating that seed dormancy was broken by warm temperatures between 21 and 29 October after seed maturation, except in 'Kitawasesoba' (whose germination rate was already as high as 96% on 21 October).

KY29, however, was derived from 'Kanoya-Zairai' × 'Hitachiakisoba.' 'Kanoya-Zairai' is an autumn-ecotype landrace grown in the warmer southern regions of Japan. It had higher PHS tolerance than the other cultivars, as previously reported (Hara et al. 2008). As 'Hitachiakisoba' did not show high PHS tolerance, 'Kanoya-Zairai' may have PHS tolerance genes and would be the donor of tolerance genes in KY29.

2. Genetic information on PHS tolerance

Buckwheat is self-incompatible, making it difficult to obtain genetic information through genetic analysis due to the difficulty of producing segregating lines. We recently developed SC lines and performed genetic analysis for some agricultural traits (e.g., Katsu et al. 2017, Matsui et al. 2007, 2008, 2018, Takeshima et al. 2019). Here, we analyzed the PHS tolerance genes of KY28 and KY29 in populations derived from crosses with the SC line KSC7, which had a fairly high germination rate. The segregation patterns of each population differed; major genes related to germination were recessive in the population derived from KY28 and dominant in that derived from KY29, indicating different tolerance genes in each line.

Our results indicate that there are at least two different mechanisms regulating the level of PHS in buckwheat, one dominant and the other recessive. In barley, two major QTLs for seed dormancy (*Qsd1* and *Qsd2*) have been identified (Han et al. 1996). Isoenzymes derived from *Qsd1*, encoding alanine aminotransferase, determine both long and short seed dormancy (Sato et al. 2016). Moreover, a gene controlling strong seed dormancy in wild barley shows maternal inheritance with incomplete dominance (Nakamura et al. 2017). However, it is still unclear whether the mechanism of seed dormancy in buckwheat is controlled by genes similar to those in barley.

KSC7 is an intermediate autumn ecotype and grows poorly in summer cropping; some progeny derived from it did not produce seeds, and we could not investigate PHS tolerance in summer cropping of the F_2 segregating population developed from it. For further analysis in summer cropping, we need to develop an F_2 segregating population from a SC summer ecotype line. Moreover, we could not evaluate the effect of xenia on PHS, so we must assess it by using other methods, because all pin plants of each F_2 progeny were open pollinated. Further study is needed to clarify the effect of xenia on PHS and these loci.

3. Breeding for PHS-tolerant cultivars and related cropping

KY28 and KY29 showed high PHS tolerance, yet 20% of seeds still germinated within seven days, thereby suggesting that PHS depends on the environment to some extent. As the expected temperature rise with global warming will enhance the risk of PHS, we need to develop cultivars with higher tolerance. The accumulation of several genes with both major and minor effects, such as those in KY28 and KY29, will be important. Pyramiding of these tolerance genes would be useful, although we do not know the tolerance mechanisms of either line. For efficient pyramiding, molecular markers linked to these genes must be developed. As tolerance in both lines seems to be controlled by several genes, QTL analysis would be a good way to develop such selection markers.

Finding new PHS tolerance alleles is also valuable. *F. homotropicum* has strong seed dormancy (Wang & Campbell 2000). We have developed lines from *F. esculentum* × *F. homotropicum* crosses, several of which have low germination rates. However, most of those crosses including KSC7 show poor PHS tolerance, so genes for strong seed dormancy were probably lost during accelerated selection. To find new PHS tolerance alleles in *F. homotropicum*, we should use germination tests or linked DNA markers, which have yet to be identified. As *F. homotropicum* has brittle pedicels (Matsui et al. 2003), and using it in conventional breeding requires much effort to eliminate the brittleness. KY28 and KY29, without brittle pedicels, would be useful for this purpose.

Gene pyramiding will make it possible to develop new cultivars with higher tolerance to PHS. However, it will be important to develop a method of breaking seed dormancy, because farmers often use seeds harvested in spring cultivation for summer cultivation. Seed dormancy can be broken by high temperatures (Samimy 1994), but we do not yet know whether the higher tolerance of newly developed lines can be broken in the same way. To use tolerance genes derived from *F. homotropicum*, it will be important to develop a method of breaking seed dormancy.

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