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INTRA-CALLUS VARIABILITY OF RICE DOUBLED HAPLOIDS GENERATED THROUGH in vitro ANDROGENESIS

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Abstract

In vitro androgenesis is among the leading methods in creating source material for crop breeding. Many breeders a priori consider the seed progeny of any doubled haploid a new line, regardless of which callus the line was obtained from. In practice, it often turns out that in field conditions the lines from one callus are outwardly identical, so the breeders discard them, leaving two or three of ones for further work. The validity of such a controversial approach requires experimental confirmation or refutation of polymorphism and genetic variability of doubled haploids of the same callus line. About 100 genes of rice resistance to *Pyricularia oryzae* Cav. [*Magnaporthe grisea* (Hebert Barr.)] are known of which *Pi-ta* и *Pi-ta*^{2} are the most relevant for the Russian Far East. This paper is the first to report intracallus morphological polymorphism and genetic variability for *Pi-ta* и *Pi-ta*^{2} genes due to gametoclonal and somaclonal variability of rice *Oryza sativa* L. doubled haploids derived from a hybrid plant via in vitro androgenesis. For the first time, a monomorphism in the absence of genetic variability due to callus cell clonal reproduction (mitotic division) was revealed among doubled haploids produced by the same callus line. Our work aimed to study the intracallus morphological and genetic variability of *Oryza sativa* L. doubled rice haploids derived in vitro androgenetically from a hybrid plant. Experiments were performed in 2017-2018 at the Federal Research Center of Agricultural Biotechnology of the Far East (Russia) and the Crop Resources Institute, Heilongjiang Academy of Agricultural Sciences, (China). A F_1 Don 4237 × Dolinnyi rice hybrid was used. A total of 386 anthers of this hybrid were cultured in vitro with the callus formation rate of 17.1%. Six of eighteen callus lines producing green regenerants were selected for further study. Two seeds of each doubled haploid line were sown in soil and grown in plastic pots (a culture room, 24 °C, 5000 lux, 16 hours/8 hours day/night). One plant of each pair was cut 90 days after sowing to assess the presence/absence of anthocyanin coloration. Plant growth stages (late tillering, stem extension, heading, and flowering) were recorded. Doubled haploids that reached the first two of these stages were assigned to a later group, and those that reached the heading and flowering stages were combined into an early group. The presence or absence of awns, their length and color were estimated after maturation. DNA was extracted from fresh leaves by CTAB method. The DNA quality and quantity were estimated electrophoretically using a 1.0 % agarose gel. Alleles of the *Pi-ta* and *Pi-ta*^{2} genes which determine blast resistance were detected by PCR method. Plants of the Chinese monogenic variety K12 were a positive control for *Pi-ta* gene, and varieties K27 for *Pi-ta*^{2} gene. The doubled haploids of two callus lines, 7.2.2 and 21.2.1, are

monomorphic without genetic variability for both resistance genes, *Pi-ta* and *Pi-ta*^{2}. Plant seeds of each callus line (7.2.2 and 21.2.1) will be further combined into two separate breeding lines to intensify breeding due to a larger number of seeds. Doubled haploids of the callus lines 1.2.1, 4.1.2, 8.2.1, 36.2.3 are polymorphic in awn formation, plant growth stage and anthocyanin pigmentation of stem cuts. The callus line 8.2.1 is genetically variable (ten plants carried alleles of both resistance genes, *Pi-ta* and *Pi-ta*^{2}, eight plants possessed none of the alleles, and in 26 plants only *Pi-ta*^{2}allele was detected). Thus, upon callus formation via in vitro androgenesis, the two opposite processes occurred are somaclonal variation and cell cloning. Somaclonal variation leads to polymorphism of callus cells and plant-regenerants, while cell cloning determines intra-callus uniformity, as well as the uniformity of some regenerants (and, in many cases, all regenerants derived from the same anther). Monomorphic doubled haploids, after a preliminary assessment for morphological traits and molecular characteristics, comprises a single selection sample.

Keywords: *Oryza sativa*, in vitro androgenesis, intra-callus variability, rice blast, resistance, *Pi-ta*, *Pi-ta*^{2}

In vitro androgenesis is one of the leading methods for creating breeding material of many agricultural crops [1-3], including rice *Oryza sativa* L. [4, 5]. Under appropriate conditions, it is relatively easy to obtain a significant number of doubled haploids, constant in morphological and genetic characteristics, with no segregation due to their homozygosity, thus the selection period becomes several years shorter [1, 4, 6]. When characterizing doubled haploids, researchers usually reveal their variability between varieties and hybrids [5, 7]. The issues of intravarietal/intrahybrid and intracallus variability remain poorly understood. According to the complex of quantitative characters in the rice cultivar Kaskad, significant differences were shown between the doubled haploids of two callus lines obtained from two anthers [9].

It is theoretically believed that one anther cultured in vitro can produce more than 1000 haploid plants [10)] which are clones [11, 12]. On one callus line of rice, tens [13] and sometimes more than a hundred doubled haploids appear. In this case, all doubled haploids of one callus line could be combined into one breeding line, which would speed up the breeding process by increasing seed pool per sample. Wheat as an example has convincingly proved the origin of androgenic embryoids from one cell [14]. It is known that there is genomic variability of rice regenerants (haploids—doubled haploids—tetraploids) within one callus line of derived from one anther [13]; therefore, genetic variability among doubled haploids is also probable. Many breeders a priori deem the seed progeny of any doubled haploid to be a new line [8, 15], regardless of which callus it was derived from. However, in the field, lines from one callus often look the same and are discarded, and only two or three of them are finally involved in further work. The validity of such a contradictory approach requires experimental confirmation or refutation of polymorphism and genetic variability of doubled haploids of one callus line.

About 100 genes of *Pi* family are known in rice for resistance to fungal pathogen *Pyricularia oryzae* Cav. [Magnaporthe grisea (Hebert Barr.)] [16] of which many are involved in breeding programs worldwide correspondingly to the pathogen race specificity. *Pi-ta* and *Pi-ta*^{2} genes are the most relevant for the Russian Far East [17]. Molecular markers allow their identification in rice plants [18-20].

This paper is the first to reveal intracallus morphological polymorphism and genetic variability for *Pi-ta* и *Pi-ta*^{2} genes due to gametoclonal and somaclonal variability of rice *Oryza sativa* L. doubled haploids derived from a hybrid plant via in vitro androgenesis. Also, there is the first demonstration of monomorphism of the doubled haploids of the same callus line without genetic variability, due to

clonal reproduction (mitotic division) of callus cells.

The work aimed to assess intracalus morphological and genetic variability of rice *Oryza sativa* L. doubled haploids generated via a hybrid plant in vitro androgenesis.

Materials and methods. Experiments were carried out in the Chaika Federal Research Center of Agricultural Biotechnology of the Far East (Russia) and the Crop Resources Institute, Heilongjiang Academy of Agricultural Sciences (China) in 2017-2018. The plant used to produce doubled rice haploids was Don 4237 × Dolinny F_1 hybrid in which a significant intracalus awnedness variability of doubled haploids was preliminaryly revealed in the R_1 regenerants. The Dolinny rice variety harbors alleles of *Pi-ta* and *Pi-ta*^{2} genes for the rice blast resistance [21, 22]. The technique for doubled haploid production was described earlier [23]. In each callus line (6 lines in total), from 9 to 44 seeds of doubled haploids of the first generation R_1 (106 lines of doubled haploids in total) were collected. For each line, two seeds per plastic pots with soil were planted and grown in a culture room at 24 °C, 5000 lx and 16 h (day)/8 h (night) mode. In the R_2 regenerants emerged from these seeds, the variability of morphological characters and growth phases were assessed.

One plant from each line of doubled haploids R_2 (106 plants in total) was cut off 90 days after planting, and the color of the cut indicative of the presence/absence of anthocyanin pigment was recorded. Leaves were collected from the same plant for DNA isolation. For another plant of the line, the following phases of growth were noted: late tillering (a straw without a flag leaf, the panicle is not visible inside the straw), stem extension (a straw with a flag leaf, the panicle is visible inside the straw), heading (5-6 upper panicle flowers), flowering (the panicle is fully visible). Double haploids that reached the first two stages were assigned to the late group, and those that reached heading and flowering were assigned to the early group. After maturation, discrete traits were assessed, that is, the presence or absence of awns, their length (short when less than 1 cm in size or as spines on some caryopses of panicles, long when more than 2 cm) and color (white black).

DNA was extracted from fresh leaves by the CTAB method [24]. The DNA quality and concertation were determined electrophoretically in 1.0% agarose gel with DNA of known concentration as a standard. Alleles of the *Pi-ta* and *Pi-ta*^{2} genes, encoding resistance, were identified by polymerase chain reaction (PCR) with the primers 5′-AGCAGGTTATAAGCTAGGCC-3′ and 5′-CTACCAACAAGTTCATCAAA-3′ for *Pi-ta* [18] and 5′-CAGCGAACTCCTTCGCATACGCA-3 and 5′-CGAAAGGTGTATGCACTATAGTATCC-3′ for *Pi-ta*^{2} [20]. The reaction mixture (20 μl) contained 2× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.25 μl each of forward and reverse primers, 1 unit of Taq DNA polymerase (Takara, Japan) and 70-120 ng of template DNA. Amplification was run in 3-fold repetition (a Veriti 96-Well Thermal Cycler, Applied Biosystems, USA). The following modes were used: 5 min at 94 °C (initial denaturation); 1 min at 94 °C (denaturation), 1 min at 56 °C (primer annealing), 1 min at 72 °C (elongation) (35 cycles); 10 min at 72 °C (final elongation) for *Pi-ta*, and 5 min at 95 °C (initial denaturation); 1 min at 95 °C (denaturation), 1 min at 60 °C (primer annealing), 1 min at 72 °C (elongation) (35 cycles); 10 min at 72 °C (final elongation) for *Pi-ta*^{2}.

Amplification products were separated electrophoretically in 1.0% agarose gel with 0.5× TBE buffer (a Sub Cell Model 192 chamber, Bio-Rad, USA; a

PowerPac Basic power supply, Bio-Rad, USA), stained with a 1.0% solution of ethidium bromide and visualized in ultraviolet light (the Gel Doc XR + gel documentation system, Bio-Rad, USA). The presence of the resistance allele was recognized only in the case of bright staining of DNA samples.

The Chinese monogenic cultivar K12 was a positive control for *Pi-ta*, and the cultivar K27 was a positive control for *Pi-ta²*.

Results. In vitro culture was generated from 386 hybrid anthers. Calli were formed in 17.1% of the anthers. Eighteen callus lines (27.3%) produced green regenerants. Six callus lines with numerous doubled haploids were finally selected for the experiment (Table 1).

1. Regeneration capacity of rice (*Oryza sativa* L.) callus lines derived from a Don 4237 × Dolinny F_1 hybrid plant via in vitro androgenesis

Callus line	Green regenerants, *n*	Doubled haploids		Sample size (R_2), *n*
		n	%	
1.2.1	138	24	17	12
4.1.2	34	28	82	9
7.2.2	16	15	94	11
8.2.1	69	57	83	44
21.2.1	24	19	79	15
36.2.3	76	71	93	15

Molecular marking revealed that electrophoretic patterns of amplification products in the paternal cultivar Dolinny and the initial hybrid plant have a band characteristic of the *Pi-ta* allele (1042 bp), whereas in the maternal cultivar Don 4237 this DNA fragment is absent (Fig. 1).

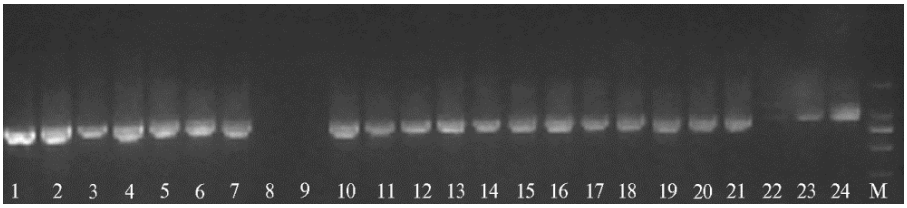

Fig. 1. Electrophoretic identification of amplicons characteristic of blast resistance gene *Pi-ta* in the parental rice (*Oryza sativa* L.) Don 4237 and Dolinny varieties, their F_1 hybrid and doubled haploids R_2 of six callus lines derived from the F_1 hybrid plant: 1, 2 — 1.2.1; 3, 4 — 4.1.2; 5, 6 — 7.2.2; 7-16 — 8.2.1, 17, 18 — 21.2.1; 19, 20 — 36.2.3; 21 — Dolinnyi variety, 22 — Don 4237 variety, 23 — Don 4237 × Dolinnyi F_1 hybrid, 24 — positive control (1042 bp, the monogenous line K12), 25 — a molecular-weight size marker D2000 («TIANGEN», China).

The *Pi-ta²* allele was found in both parental forms and in the hybrid. Doubled haploids of five callus lines (1.2.1, 4.1.2, 7.2.2, 21.2.1, and 36.2.3) had alleles for both *Pi-ta* and *Pi-ta²* resistance genes (Table 2). Doubled haploids derived from the callus line 8.2.1 showed genetic variability, i.e. 10 plants harbored alleles of both resistance genes, in 8 plants both alleles were absent, and in the remaining 26 plants only the *Pi-ta²* allele was revealed. The peculiarities of differentiation of the callus line 8.2.1-derived doubled haploids had little effect on the presence of detectable amplification products. Thus, out of 69 green regenerants formed (see Table 1), plants with alleles of both resistance genes were found among the R_2 doubled haploids Nos. 1-44, of only *Pi-ta²* among the R_2 doubled haploids Nos. 22-68. Note, alleles of both resistance genes were absent in a part of the doubled haploids Nos. 2-69, thence, no definite regularity was observed.

An analysis of phenotypic variability showed that the doubled haploids of callus lines 7.2.2 and 21.2.1 are monomorphic. Polymorphism was observed among the doubled haploids of four callus lines. The callus line 8.2.1 showed the greatest morphological variability, and this variability was not associated with the genetic

variability of doubled haploids (see Table 2).

2. Genotypes and phenotypes of rice (*Oryza sativa* L.) doubled haploids R_2 of six callus lines derived from a Don 4237 × Dolinny F_1 hybrid plant via in vitro androgenesis

Callus line	Doubled haploids, *n*	Stem cut color	Awns, size	Type of development	Genes	
					Pi-ta	*Pi-ta²*
1.2.1	2	Anthocyanin	Short	Early	+	+
	1	Anthocyanin	Absent	Early	+	+
	6	Anthocyanin	Short	Late	+	+
	3	Anthocyanin	Long	Late	+	+
4.1.2	2	Anthocyanin	Short	Early	+	+
	4	Anthocyanin	Short	Late	+	+
	1	Anthocyanin	Absent	Early	+	+
	2	Anthocyanin	Absent	Late	+	+
7.2.2	11	No anthocyanin	Long	Late	+	+
8.2.1	3	Anthocyanin	Short	Early	+	+
	2	Anthocyanin	Long	Late	+	+
	1	No anthocyanin	Long	Late	+	+
	3	Anthocyanin	Absent	Early	+	+
	1	No anthocyanin	Absent	Early	+	+
	1	Anthocyanin	Short	Early	−	+
	1	Anthocyanin	Long	Early	−	+
	18	Anthocyanin	Short	Late	−	+
	1	Anthocyanin	Long	Late	−	+
	5	No anthocyanin	Short	Late	−	+
	1	Anthocyanin	Short	Early	−	−
	5	Anthocyanin	Short	Late	−	−
	1	No anthocyanin	Short	Early	−	−
	1	No anthocyanin	Short	Late	−	−
21.2.1	15	Anthocyanin	Absent	Early	+	+
36.2.3	2	Anthocyanin	Absent	Early	+	+
	13	Anthocyanin	Absent	Late	+	+

N o t e. «+» — presence of the resistance gene; «−» — absence of the resistance gene.

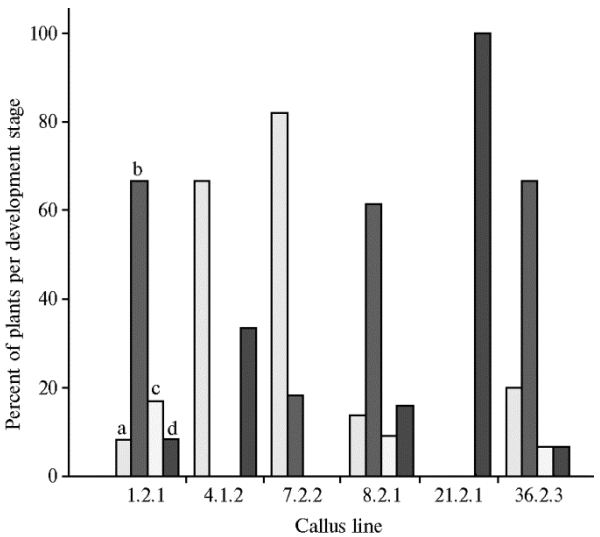

Fig. 2. Distribution of rice (*Oryza sativa* L.) doubled haploids R_2 of six callus lines derived from a Don 4237 × Dolinnyi F_1 hybrid plant via in vitro androgenesis according to the growth stage 90 days after planting: a — tillering, b — stem extension, c — heading, d — flowering.

Figure 2 shows the detailed distribution of doubled haploids according to the stage of growth. All plants of callus line 21.2.1 flowered, no variability in other traits was observed either. Plants of line 4.1.2 were clearly divided into two groups, three early (in the flowering stage) and five late (in the tillering stage), among those and others were those awned and awnless (see Table 2). Plants of line 7.2.2 were late in the timing of both tillering and stem extension stages and monomorphic in other traits. In three of the six callus lines (1.2.1, 8.2.1, and 36.2.3), all four growth stages were observed in doubled haploids. Of these, line 36.2.3 is completely awnless with anthocyanin coloration of the straw cut, that is, variable only in early maturity, line 1.2.1 is polymorphic in two traits, the awn formation and growth stages. In line 8.2.1, doubled haploids exhibited both genetic and phenotypic variability, and among the doubled haploids, a different combination of morphological and genetic traits was revealed (see Table 2). We did not observe variability in the color of

awns among doubled haploids of the same callus line, i.e. in line 7.2.2, the awns of caryopses were white, in lines 1.2.1, 4.1.2, and 8.2.1 they were black

The anther of rice contains about 1,000 pollen grains [25] which can be induced into callus. The callus line from one anther derived from callus aggregates, which can be formed by several immature microspores. This leads to both polymorphism and genetic variability among doubled haploids and to genomic variability of regenerates of one callus line (haploids, doubled haploids, and tetraploids) [13]. However, clonal reproduction of regenerants in vitro is also not excluded. Thus, for some morphotypes and genotypes, there are from a small number to several tens of doubled haploids, i.e. 18 plants in the callus line 8.2.1 (see Table 2). Earlier, we detected the formation of up to 7-18 tetraploid plants of one callus line. Although the polymorphism of tetraploids of one callus line has not been studied, their massive formation on one callus most likely occurs due to mitotic the replication of 4*n* callus cells [26]. The doubled haploids of callus lines 7.2.2 and 21.2.1, which are monomorphic, can be considered as identical plants and combined into two separate sets during selection. Similar methodology is obviously applicable for groups of identical plants within other callus lines.

The callus line 8.2.1 is of particular interest due to emergence of various regenerant types, i.e. those with alleles of both resistance genes, *Pi-ta* and *Pi-ta*^{2}, having only *Pi-ta*^{2} and not carrying alleles of any of these genes. There are several possible explanations for such a segregation of the line 8.2.1 regenerants. One of the parents (variety Dolinnyi) is a carrier of the *Pi-ta* allele which determines resistance, and his hybrid inherited this gene in a heterozygote. As a result, some of the doubled haploids had the *Pi-ta* allele, and some did not (see Fig. 1). Both parents and the hybrid possessed the *Pi-ta*^{2} allele, but it was absent in 8 doubled haploids (see Table 2). The primers we used allow detection of the presence or absence of an allele of the resistance gene but do not reveal the allelic state of the gene. It can be assumed that one of the parents (variety Don 4234) is heterozygous, and the other (variety Dolinnyi) is homozygous for *Pi-ta*^{2} gene, while the hybrid is heterozygous for this gene. Figure 3 shows a possible scheme of dihybrid crossing which leads to genetic variability among the doubled haploids of callus line 8.2.1. Only the second variant of crossing provides for three types of combinations of resistance alleles in regenerated plants. *AB*, *aB*, and *ab* microspores induced callus formation, followed by spontaneous chromosome doubling and regeneration. Microspores of the fourth type *Ab* either did not generate a callus, or only produced haploids.

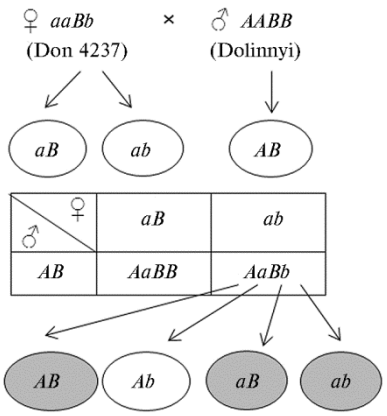

Fig. 3. A possible scheme for the dihybrid crossing of rice (*Oryza sativa* L.) parental varieties, followed by callus formation from microspores to produce doubled haploids with two alleles of the blast resistance genes *Pi-ta* and *Pi-ta*^{2}, with one allele (only for *Pi-ta*^{2}) and without alleles of both resistance genes: A — *Pi-ta* allele which determines resistance, a — *Pi-ta* allele which determines susceptibility, B — *Pi-ta*^{2} allele which determines resistance, b — *Pi-ta*^{2} allele which determines susceptibility.

Wang et al. [27] consider the *Pi-ta* and *Pi-ta*^{2} genes to be allelic or closely linked to each other. According to Oryzabase [28], this is the same gene of 7281 nucleotides in size detected by different primers that amplify different parts of the gene. *Pi-ta* gene allele polymorphisms have been studied in several rice species and varieties. Only 99% of alleles are found to be similar in

nucleotide sequence and show not identical level of divergence [29]. True, the phenotypic manifestation of *Pi-ta* and *Pi-ta²* genes, revealed with the use of differentiating varieties, differs significantly [17, 20, 30]. In the studied plants, one could expect either the presence or the complete absence of resistance alleles for both genes. However, we have revealed in 26 plants only the *Pi-ta²* gene allele which determines resistance.

The phenomenon of somaclonal variability is widespread in cell and tissue cultures in vitro, it causes an increase in genetic variability and often does not affect the viability of regenerants derived from calli [31-33]. In our experiment, genetic mutations caused by in vitro culture are not excluded. These changes could have occurred in the DNA region of the *Pi-ta* gene, which is not identical to the *Pi-ta²* gene, in callus cells even before the spontaneous doubling of chromosomes followed by formation of doubled haploids. It is impossible to exclude mutations in DNA regions that led to the absence of resistant alleles of both genes in 8 doubled haploids. This is supported by the presence of the resistant alleles of *Pi-ta* and *Pi-ta²* among the doubled haploids of 5 other callus lines. Possibly, due to somaclonal variability that arose after spontaneous doubling, several samples could harbor any of the studied genes in a heterozygous state, which cannot be detected using the primers chosen.

The revealed polymorphism may indicate that in the anther, which was used to obtain line 8.2.1, callus formation was induced in at least 14 immature microspores, followed by chromosome doubling and regeneration. However, a high frequency of physiological and quantitative changes among somaclones was previously noted, such as the time of flowering (panicle emergence), ripening time, presence or absence of awns, plant height [32, 34]. Morphological, biochemical, and molecular genetic analysis showed that during somaclonal variation, already at early culture stage, similar genetic transformations occur, leading to the appearance of common characters in different groups of somaclones of the same cultivated species [35]. The coincident variability can be explained by transposition explosions [34]. Among the tobacco and rapeseed dihaploids, morphological mutants segregate, which must be completely homozygous [34]. If such variability occurs in the doubled haploids of the four callus lines obtained by us (1.2.1, 4.1.2, 8.2.1, 36.2.3), genetic changes are possible even at the stage of haploid cells with subsequent spontaneous doubling. The other two callus lines not affected by this variability are probably more stable (7.2.2, 21.2.1). Therefore, it can be assumed that the ability to accumulate mutational changes in cell in vitro culture is significantly influenced not only by the variety of the original plant, which was established by Kuznetsova et al. [33], but also by the genotype of the pollen grain used for in vitro androgenesis.

In vitro androgenesis allows production of a huge variety of doubled haploid lines for breeding programs. In particular, the combination of monomorphic doubled haploids of one callus line into a single breeding line accelerates breeding due to the increased number of seeds per sample. It is also possible to use the diversity of doubled haploids observed in some *O. sativa* callus lines, considering each of them as a separate breeding line. This is especially important for genotypes with a hindered androgenesis in vitro: even upon callus formation induced only in one of the many immature anthers, the derived callus line allows one to obtain several polymorphic lines of doubled haploids.

Next, we plan to study the resistance of the obtained early-maturing leafless lines of doubled rice haploids carrying alleles of both genes, *Pi-ta* and *Pi-ta²*, upon artificial infection with *P. oryzae* strains circulating in the Far Eastern rice

growing zone.

Thus, we have revealed for the first time intracallus morphological polymorphism and genetic variability for *Pi-ta* and *Pi-ta²* blast resistance genes in rice (*Oryza sativa* L.) doubled haploid lines obtained via in vitro androgenesis from one hybrid plant. The doubled haploids of callus lines 7.2.2 and 21.2.1 are monomorphic and do not show genetic variability for both resistance genes, *Pi-ta* and *Pi-ta²*. The doubled haploids of four other callus lines (1.2.1, 4.1.2, 8.2.1, and 36.2.3) are polymorphic in awnedness, plant development rate, and anthocyanin coloration of cuts. The callus line 8.2.1 is genetically variable and represented by three types of plants, those with alleles of both *Pi-ta* and *Pi-ta²* genes, only the *Pi-ta²* gene allele, or without alleles of both genes. Callus formation during in vitro androgenesis involves two-direction events. The first is the well-studied scenario of somaclonal variation which causes polymorphism of cells or regenerants, and the second is cell cloning which leads to intracallus homogeneity, the homogeneity of some of the regenerants, and in many cases of all the regenerants derived from one anther. The monomorphic doubled haploids, after a preliminary morphological and molecular genetic characterization, are grouped into one sample to be further involved in the breeding program.

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