

***K-Plus*: A Gene Controlling Potassium Content in a Light-green Wheat Mutant**

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A wheat (*Triticum turgidum* L. var. *durum*) mutant, designated *k-plus*, constitutively possesses high leaf potassium content and light-green leaves. To study the genetic basis of these traits and to find out their association, *k-plus* mutant was crossed to the wild type (WT) Trinakria, *F1* and *F2* populations were developed. The flag leaves of parental lines, *F1* and *F2* progeny were assayed for ion content, at the heading stage. Potassium content was greater in *k-plus* than in WT; the *F1* hybrids behaved like to the WT, in both potassium and leaf colour. The number of genes conditioning leaf potassium content, was not more than one, as estimated by Castle–Wright method. A single recessive locus controls potassium content of *k-plus* mutant, with the wild type allele completely dominant over the new mutant. Broad sense heritability can be considered sufficient to obtain progress from selection, of *k-plus* phenotype. Light-green leaf colour was inherited as monogenic recessive allele, which co-segregate with the locus controlling potassium accumulation. Light-green *F2* sub-population had, on the average, significantly more potassium content than green *F2* sub-population. These preliminary results encourage further physiologic and genetic analysis of *k-plus* mutant.

Keywords: potassium, mutant, genetic analysis

Introduction

The deleterious effects of abiotic stress on yield represent an unsolved trouble that strongly hampers the satisfaction of worldwide growing cereal demand. Potassium is a macronutrient essential for stress-related physiologic functions like osmoregulation, cell expansion, leaf turgor, stomatal activity, enzyme activation etc. Physiology of potassium uptake has been widely studied, considering its importance to maintain ion homeostasis and to characterize the ionome in higher plants (Salt 2004). In durum wheat potassium ion was the major inorganic contributor to osmotic adjustment, also capable to exert an effect on the binding-strength of tightly bound water (Rascio et al. 1994).

Ionic content within the leaf will depend upon the rate of solute supply, the rate of solute export and the extent of solute uptake and retention (Karley et al. 2000) and capacity for discrimination between K^+ and Na^+ , was typically associated with the salt tolerance.

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Multiple genes and even multiple gene families, encoding plant K^+ channels and transporters have been identified (Shabala 2003; Platten et al. 2006; Grabov 2007). Quantitative trait loci (QTL) involved in uptake and distribution of K^+ in plants are known (Wu et al. 1998; Harada and Leigh 2006; Lebaudy et al. 2007), however there is minimal information on the genetic basis and on feasibility of selection for potassium content in wheat. Potassium accumulation in rice and in forages resulted heritable and under genetic control (Hill and Guss 1976; Garcia et al. 1997). Additive gene action was indicated for concentration of potassium in *Zea mays* ears and leaves (Gorsline et al. 1964). In monocots a single gene (*Kna1*) controlled the difference, between the disomic substitution line 4D (4B) and durum cv 'Langdon' (Dubcovsky et al. 1996), whereas two genes were detected in material derived from *Triticum monococcum* (Munns et al. 2003).

To rapidly discover the physiological role of stress-related gene sequences (Cattivelli et al. 2002), to understand the genetic basis of physiologic traits and to obtain sources of germplasm, from which alleles can be rapidly and efficiently incorporate into other germplasm, mutants for mineral content have been identified (Saleki et al. 1993; Delhaize 1996). The combination of genetic analyses and molecular tools has greatly facilitated the molecular cloning of plant genes and has accelerated the transfer of useful genes in breeding programs. In wheat, selection for increased potassium concentration of the leaves was successfully employed to isolate a light-green mutant, for which we propose the name: *k-plus* (for potassium accumulator), that constitutively had about 5 mg K^+ /g dry weight (DW), more than WT, in the leaves (Rascio et al. 2001). Mutagenesis generally has unearthed salt-sensitive types (Flowers 2004; Zhu et al. 2007), while *K-plus* displayed

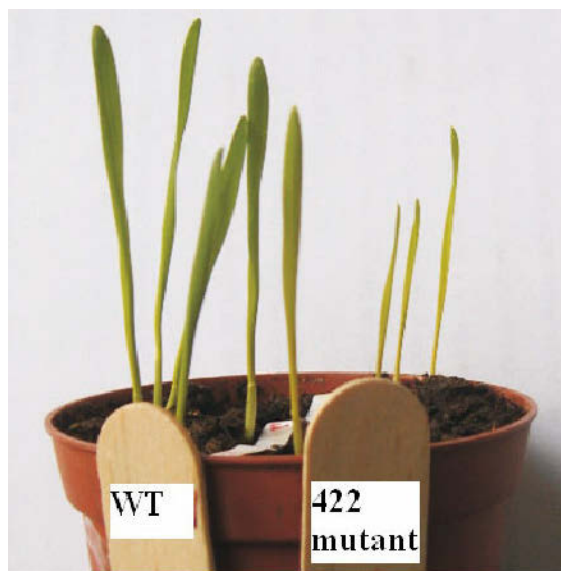


Figure 1. Comparison of leaf colour of Wild Type (on the left) and *K-plus* (on the right). Representative seven-day-old plants

greater tolerance than wild type to osmotic stress and it is characterized by an apparent light-green colour (Fig. 1) that could be a phenotypic marker to make easier selection. This study was designated to characterize preliminarily the durum wheat *K-plus* mutant and to verify if the light-green trait can be used as a morphological marker, to facilitate selection for high potassium content.

Materials and Methods

Mutants were obtained as previously described (Rascio et al. 2001). Seeds of durum wheat (Trinakria cultivar) were mutagenized using sodium azide, one seed of each of 4000 mutagenized plants was sown in field and plants were selfed for two generations. The youngest fully expanded leaf from 270 selected M4 plants was collected at different growth stage, to measure K^+ concentration by atomic absorption. Several plants with abnormal K^+ concentration in the leaves were identified and their seeds were collected (M5 lines). Individual M5 plants, from 16 putative mutants were grown in the field to analyze the accumulation kinetic of potassium. The *k-plus* mutant – named 422 line in a previous work (Rascio et al. 2001) – was selected and characterized in further studies, conducted in a controlled environment and in the field (Rascio et al. 2001).

To determine the mode of potassium inheritance, *k-plus* mutant (used as the male) was crossed with the Trinakria wild type (WT), by hand emasculation and the resulting cross *F1* was selfed, to produce the *F2* generation. During 1997 and 1998 years, *k-plus* mutant, the wild type, their *F1* hybrids and the *F2* population were sown, in the field of Research Center for Cereal Crops of Foggia. The experiments consisted of a randomized complete block design, with three replications. Six rows of *F2* plants, three rows of Trinakria cv., three of *k-plus* mutant and one row of the *F1* were evaluated per replication. Rows were 30 cm apart and plants in each row were thinned, to obtain similar plant density. Plants were fertilized at stem elongation with 300 kg ha^{-1} of ammonium nitrate and were grown without irrigation. No disease attacks were noted. Flag leaves were collected from 15–20 plants of each parental or the *F1* and from more than 200 plants (about 80 plants for each replication) of *F2* generation.

During each year potassium content of the flag leaf was determined, by atomic absorption, at heading stage: tissues were dried for 18 h in a vacuum oven at 80°C , weighed and ashed at 550°C for 3 h. Inorganic ions were extracted with 10 ml, 1 M H_2SO_4 . Concentration of K^+ , Na^+ , Ca^{++} , Mg^{++} in these solutions was measured, using a Perkin Elmer 370A spectrophotometer.

Statistical analysis

On the individual data, for each generation, analyses of variance were done to test for differences among genotypes. *T test* and *Tukey's* least significant difference test were used for mean comparisons. Regression analysis was outlined to test association between categorized leaf colour and potassium content.

The number of genes involved in the leaf K^+ content was estimated using the Castle–Wright formula (Castle and Wright 1921)

$$N = (K\text{-}plus_{\text{mean}} - WT_{\text{mean}}) / 8(V_{F1} - V_{F2})$$

where V_{F1} and V_{F2} are the variances among plants in the $F2$ and in the $F1$ populations.

Normality of $F2$ distributions was tested by Kolmogorov–Smirnov (KS) test.

The $F2$ plants were allocated into discrete classes, according to the method of Allard (1956): standard deviations (estimated from the variance within wild type and mutant parents) were calculated, being considered the best estimate of the environmental variance of each genotype. From frequency distribution and means of parents, theoretical curves (representing the genotypes expected in $F2$ generation) were constructed by GraphPad computer software (Motulsky 1999); the frequency distributions were divided in two parts, beyond which the area under the two expected parental distribution curves were equal (arrows in Fig. 2). Chi-square analysis was used, to test if the gene model fitted to the observed data.

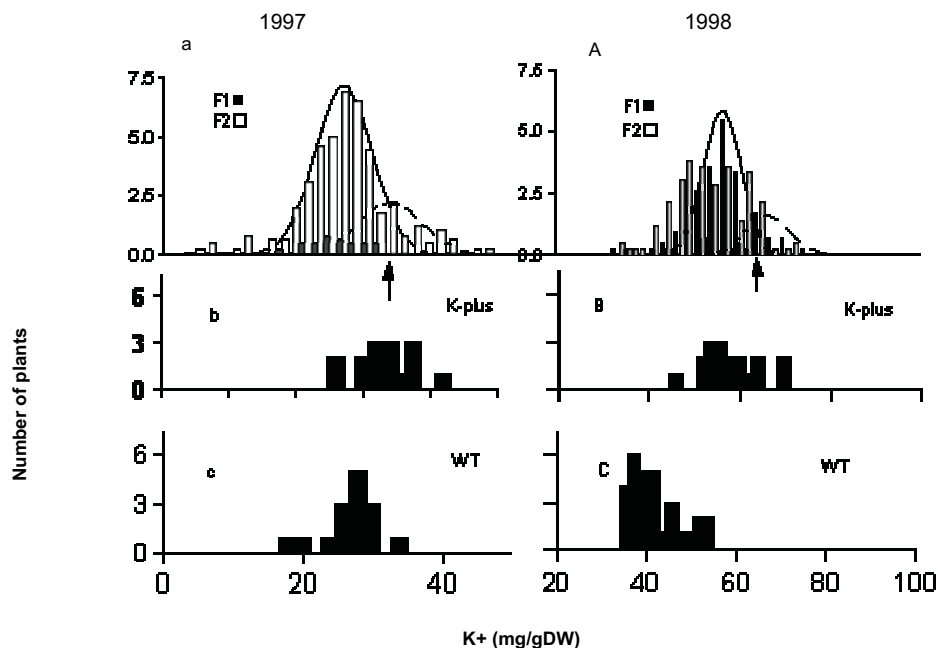


Figure 2. Vertical bars show the frequency of $F1$, $F2$ (a, A) and parental (b, B, c, C) individuals with various levels of potassium leaf content, grown in the field in 1997 and 1998. Two curves (a, A) show theoretical frequency distribution representing the dominant homozygote and recessive parents, based on monogenic segregation with complete dominance. Big arrows depict the point beyond which the area under the two theoretical distribution curves of the parents is equal to each other

Heritability (H) in the broad sense was calculated from parental, $F1$ and $F2$ variances by using the following formula:

$$H = [(V_{F2} - V_E)/V_{F2}] \times 100$$

where V_{F2} is phenotypic variance among plants in the $F2$ plants and V_E is environmental variance, as estimated by the arithmetical mean of the variance among plants of the parental cultivars and the $F1$ plants.

Results

On the average, leaf potassium content of parental genotypes changed with years. In both 1997 and 1998 significant differences ($P < 0.001$), between *k-plus* and wild-type were detected (Table 1), while genotype \times year interaction was not significant. Potassium content of $F2$ populations was highly variable, but in the previously observed (Garcia et al. 1997; Harada and Leigh 2006) natural range.

Table 1. Mean leaf potassium content and variances of parents (WT and *k-plus*) and progenies ($F1$ and $F2$) determined in field, at heading stage, in 1997 and in 1998

	K ⁺ (mg/gDW)			
	WT	<i>K-plus</i>	$F1$	$F2$
1997	26.6a	33.6b	26.5a	28.8a
Variance	17.6	20.7	11.1	29.7
N	15	15	18	225
1998	45.6a	58.0c	47.4ab	50.7bc
Variance	25.8	39.3	39.0	62.4
N	18	18	13	207

Means followed by the same letter do not differ significantly at 0.001 probability level according to Tukey's test.

Because K⁺ content of hybrids was significantly different from that of *k-plus* mutant, but did not differ from wild-type mean, the mutation was recessive.

The number of genes conditioning leaf potassium content, estimated by Castle–Wright method, was not more than one. The $F2$ frequency distribution (Fig. 2) was not normal (KS distance = 0.284, $P = 0.0416^{**}$ in 1997; KS distance = 0.272, $P = 0.0016^{**}$ in 1998), but the bimodality of the distribution, expected for a qualitative trait was not evident, because masked by the rather large effects of environmental variability. For these reasons a method for testing the number of genes affecting the inheritance of semi-qualitative characters (Allard 1956) was used as a guide, in allocating the $F2$ plants into discrete classes: parental means were assumed to represent the dominant and the recessive homozygote genotypes expected in the $F2$ generations. The $F2$ plants were allocated into discrete classes, according to the method of Allard (1956). Standard deviations (estimated from the variance within wild type and mutant parents) were calculated, being considered the best estimate of the environmental variance of each genotype. From frequency distribution and means of parents, theoretical curves (representing the genotypes expected in $F2$ generation) were constructed by GraphPad computer software (Motulsky 1999); the frequency

distributions were divided in two parts, beyond which the area under the two expected parental distribution curves were equal (arrows in Fig. 2). Chi-square analysis was used, to test if the gene model fitted to the observed data.

Theoretical curves representing the genotypes expected in *F2* generation were generated by computer software and showed in Figure 2a (1997 year) and 2A (1998 year); the arrows depicting the intersection points, where areas of the expected parental distribution curves were equal, was strictly closed to the upper limit of *F1* population. The frequencies of the resulting two parts well fitted to the 3:1 expected ratio (Table 2), as a consequence the method used to allocate the *F2* plants to different discrete classes is robust, individual plant ratios suggesting that potassium content was conditioned by a single recessive gene in *k-plus* mutant.

Table 2. Segregation ratios for *K-plus* to non-*K-plus* plants, in *F2* generations of *K-plus* × Trinakria cross and their Chi-square analyses

	1997		1998	
	<i>K-plus</i>	non- <i>K-plus</i>	<i>K-plus</i>	non- <i>K-plus</i>
Observed	181	44	254	114
Expected	169	56	276	92
Chi-square		1.85		3.26
P		0.174 ns		0.071 ns

ns = not significant

Broad sense heritability estimated in each year, was about 44% of the total variation in the *F2* and was similar to that observed for other species (Garcia et al. 1997; Harada and Leigh 2006).

The mutant self-pollinated for 6 generations was characterized by an apparent light-green colour that slightly varied for, when plant grew under different environments (Rascio et al. 2001). The same trait was not expressed in hybrids with normal green WT. Segregation of light-green colour was analysed on the *F2* population in 1998 and was as a single locus (261 wild-type: 104 light-green phenotype; $\chi^2 = 1.7$, $p = 0.19$ for a 3:1 ratio). Frequency distributions for monovalent and divalent cations in *F2* sub-populations, differing for leaf colour were compared, too (Fig. 3). The patterns of frequency distribution were very similar for sodium, calcium and magnesium ions, while distribution of K^+ content was different for light-green and green plants. *T test* clearly indicated that light-green plants had significantly more potassium than green plants (Table 3), while did not differ for content of other ions. Slope of linear regression, constructed by plotting categorized leaf colour vs potassium content was significantly different from zero, confirming that these traits are associated (Fig. 4). When monovalent ion content of the leaves was plotted versus the divalent cation content, a significant negative linear regression was observed for green plants, while no relationship was observed for light-green plants (Fig. 5).

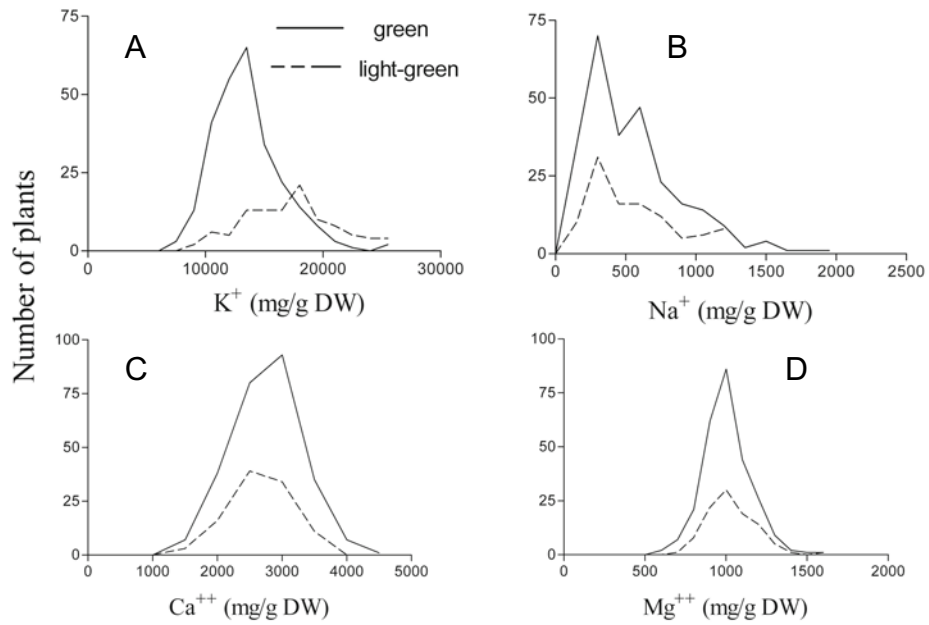


Figure 3. The frequency distribution of mono (A, B) and bivalent (C, D) cations in the green and in the light-green sub-populations of the F₂ generation, grown in the field in 1998

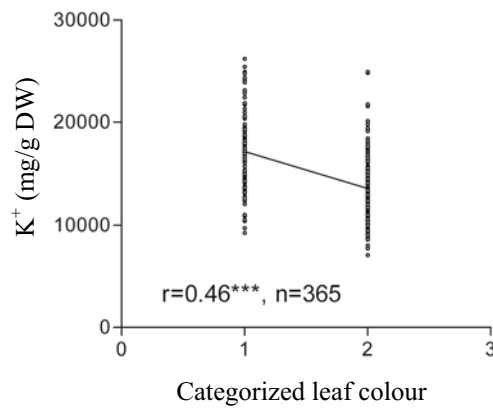


Figure 4. Potassium content of two F₂ sub-populations determined on plants grown in 1998 year, differing for leaf colour: 1 = light-green plants, 2 = green plants. Linear regression and correlation coefficient (r) are shown. ***: significant at 0,001 probability level; n = number of plants

Table 3. The mean monovalent and bivalent content of *F2* plant leaves determined at the heading stage in 1998

	Light-green	Green	Light-green	Green	Light-green	Green	Light-green	Green
	K ⁺ (ppm)		Na ⁺ (ppm)		Mg ⁺⁺ (ppm)		Ca ⁺⁺ (ppm)	
N°	104	261	104	261	101	261	104	261
Media	17144	13527	548.8	545.7	1028	1009	2656	2748
T test	9.77***		0.08 ns		1.11 ns		0.72 ns	

***: significant at 0.001 probability level; ns: not significant at 0.001 probability level

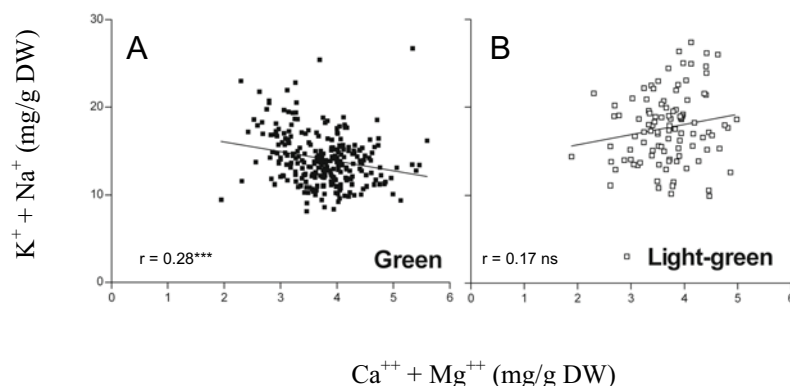


Figure 5. The relationship between mono and bivalent cation content in the green (A) and in the light-green leaves (B) of one *F2* sub-population, grown in the field, in 1998. Linear correlation coefficient (*r*) is shown. ***: significant at 0,001 probability level; ns: not significant at 0,001 probability level

Discussion

Control of K⁺ concentration involves several gene products, but whether they all are important, it is unknown. The detected loci and their number depend on genetic differences of materials compared. As an example K⁺/Na⁺ selectivity is controlled by a large number of loci and the dissection of the trait, in *Lophopyrum elongatum*, showed that virtually every chromosome controlled that trait (Omielan et al. 1991). In Arabidopsis, shoot K⁺ homeostasis resulted to be controlled by the HKT1 gene, originally isolated from wheat (Platten et al. 2006). Single gene mutation conferred salt tolerance in fern mutants with altered K⁺ transport (Warne and Hickok 1987) and it has been possible modify plant potassium status and potassium supply (Hirsch et al. 1998), through disruption of a single high affinity K⁺ transporter (AtHAK1).

Results of this preliminary study indicate that K⁺ ion content of *k-plus* leaves is recessive and most likely monogenic. About 44% of the total variation in the *F2* generation is genetic and can be considered sufficient to obtain progress, from selection for *k-plus* phenotype.

The light-green phenotype co-segregated with the locus controlling potassium accumulation and as for potassium content, the trait was recessive and was inherited as a simple trait. Light-green plants did not show the K-Na discrimination trait; in fact they had sodium ion content similar to that of green plants and only for light-green plants, monovalent ion content was not related to the bivalent content (Fig. 4). These findings suggest that the mutation may have influenced the well-known mechanisms (Shabala et al. 2005) that regulate the competitive assumption and/or deposition of divalent cations and potassium in the leaves.

Light-green colour is not necessarily the symptomatic manifestation of a pathologic plant conditions, because differing from others mutants, whose chlorotic phenotype was the consequence of excessive accumulation of metal ions (Van Vliet et al. 1995; Delhaize 1996), *k-plus* mutant had not a toxic concentrations of potassium in the leaves. Other authors (Sarić 1979), reported that changes for potassium ion content were physiologically associated to different colour intensity of *variegata* leaves, with green sections having lower K^+ . Anyway, physiological studies have been planned to ascertain that the light-green trait is not due to a defect in the assembly of the PSI or PSII antenna, with a consequent reduced photosynthetic performance. *K-plus* mutant may be potentially of interest to plant breeding and to study ion metabolism. The monogenic control of potassium content in *k-plus*, may introduce new possibility to investigate the biological function of the mutated gene and may facilitate its transfer into other genotypes. The co-segregation observed between potassium content and leaf colour indicates that the selection of near-isogenic lines differing only at genes, at the *k-plus* locus, may be simplified, using leaf colour as a phenotypic marker and looking for recombinant between *k-plus* and leaf colour in the cross involving the isogenic reselection. To this aim, it is necessary to establish whether the two traits are a pleiotropy of a single mutation or whether two different genes are affected. Confirmation of the mutant utility, suggested by these preliminary results, will require further physiological study and a molecular genetic analysis through a QTL mapping approach.

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