Research Paper

The impact of the *SSIIa null* mutations on grain traits and composition in durum wheat

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Starch represents a major nutrient in the human diet providing essentially a source of energy. More recently the modification of its composition has been associated with new functionalities both at the nutritional and technological level. Targeting the major starch biosynthetic enzymes has been shown to be a valuable strategy to manipulate the amylose-amylopectin ratio in reserve starch. In the present work a breeding strategy aiming to produce a set of *SSIIa* (starch synthases IIa) *null* durum wheat is described. We have characterized major traits such as seed weight, total starch, amylose, protein and β -glucan content in a set of mutant families derived from the introgression of the *SSIIa null* trait into Svevo, an elite Italian durum wheat cultivar. A large degree of variability was detected and used to select wheat lines with either improved quality traits or agronomic performances. Semolina of a set of two *SSIIa null* lines showed new rheological behavior and an increased content of all major dietary fiber components, namely arabinoxylans, β -glucans and resistant starch. Furthermore the investigation of gene expression highlighted important differences in some genes involved in starch and β -glucans biosynthesis.

Key Words: starch, high amylose, durum wheat, dietary fiber.

Introduction

Durum wheat represents an important crop whose main use is the production of high quality pasta products, and a wide range of different types of bread. The studies of factors determining pasta quality have mainly been addressed to the gluten proteins, whereas the role of the starch fraction has been studied to a lesser extent. Recently, a renewed interest in starch functionality has opened new opportunities for the improvement of wheat based food products: the feasibility to manipulate starch composition, with particular attention paid to the amylose/amylopectin ratio, has resulted in the possibility to develop wheat lines with further improved nutritional and technological characteristics. In particular, high amylose starch has been associated with an increased level of resistant starch (RS) a starch fraction providing several benefits for humans due to its effects similar to dietary fiber (Topping 2007). An increase in resistant starch content

lowers glycemic index of the food and helps in preventing the onset of serious diet related diseases (Lafiandra *et al.* 2014, Topping 2007). In addition, a positive effect of high amylose starch on pasta quality characteristics has been reported (Soh *et al.* 2006).

In wheat the manipulation of the key starch biosynthetic enzymes, such as starch synthases IIa and starch branching enzymes, has permitted the development of durum and bread wheat genotypes with increased amylose content (Hazard *et al.* 2015, Konik-Rose *et al.* 2007, Lafiandra *et al.* 2010, Sestili *et al.* 2015, Slade *et al.* 2012, Yamamori *et al.* 2006). *SSIIa* enzyme is known to be essential for the elongation of amylopectin linear chains and its absence result in a drastically changed phenotype in a number of cereal species (Morell *et al.* 2003). In particular, Yamamori *et al.* (2006) described the starch phenotype of a complete *SSIIa-null* bread wheat line, obtained crossing single *null* natural mutants they had previously identified after screening a large bread wheat collection (Yamamori *et al.* 2000). The work of these authors clearly demonstrated the existence of pleiotropic effects generated by the simultaneous inactivation of the three *SSIIa* homeoalleles. They found an increase in the apparent amylose content of 27% compared to the

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control plants what is the actual level of amylose, a changed amylopectin chain branching pattern and the appearance of deformed A-type granules; these characteristics were accompanied by an increased level of RS and novel starch viscosity properties. The introgression of the *SSIIa null* alleles in the Australian bread wheat variety Sunco confirmed a similar increase in amylose (29%) along with an important decrease in total starch (15–20%) (Kosar-Hashemi *et al.* 2007). Lafiandra *et al.* (2010) introgressed the *SSIIa null* alleles in the durum wheat cultivar Svevo detecting a more drastic increase of amylose content of roughly 89% in respect to the control plant. More recently, Hogg *et al.* (2013) have produced a new set of partial and complete *SSIIa null* durum wheat, by the combination of natural and chemical induced mutations. Analysis of these materials has confirmed an increase in amylose content and a drastic decrease in the total amount of starch (by 45%). Summarizing, wheat grains lacking *SSIIa* enzymes are reported to have good quality features, due to the higher amylose and resistant starch contents but poor agronomic characteristics, associated with low yield.

In the present work, we have investigated the effects associated with the introgression of *SSIIa null* mutations in the elite Italian cultivar Svevo, analyzing a large set of mutant families that were characterized for starch characteristics and seed traits. In addition, semolina produced from *SSIIa null* lines was characterized at rheological level and its composition was investigated.

Materials and Methods

Plant material

The two mutant lines Chousen 30 (*SSIIa-A null*) and Kanto 79 (*SSIIa-B null*) identified by Yamamori and Endo (1996) had previously been used to produce complete *SSIIa null* lines in the Italian durum wheat cultivar Svevo. The crossing program included a backcross of the two mutant lines. Starch granule proteins, isolated from half seeds of the two BC₁F₂ populations, were analyzed electrophoretically as described by Sestili *et al.* (2010). The plants with single *SSIIa-A* and *SSIIa-B null* were crossed and double F₂ *nulls* selected electrophoretically. F₂ plants were backcrossed with Svevo and 144 complete *null SSIIa* BC₄F₆ mutant families were recovered. Selection of the mutant families was also conducted by molecular markers designed on the mutations of the A and B homeoalleles as described in Shimbata *et al.* (2005). Fourteen durum wheat mutant lines, showing the best and worst values for grain yield, were selected and characterized in the present study. The set of fourteen lines were grown in field, following a randomized block design, in two different years (2009 and 2010) in Argelato (Bologna-Central Italy). Seed density was 400 seeds/m².

Two further lines (H795 and H806), advanced to BC₄F₉, were also grown in the same location in the 2013 and 2014 and seeds harvested were milled to produce semolina.

RNA isolation from immature seed

Total RNA was extracted from immature seeds, harvested at 21 dpa (days post anthesis), of the durum wheat cultivar Svevo and a derived *SSIIa* double *null* line (H770) as reported in Sestili *et al.* (2010). For reverse transcriptase-mediated PCR studies, cDNA was synthesized from 1 µg of total RNA using an oligo(dT) primer and Superscript Reverse Transcriptase III (Life Technologies, Grand Island, NE).

Expression analysis of genes coding the starch biosynthetic enzymes by real time RT-PCR

qRT-PCR experiments were performed as already described in Sestili *et al.* (2010). The gene Ta2526 was used as housekeeping gene. The primer pairs for genes *GBSSI* (*Granule Bound starch synthase*), *SSI*, *SSII*, *SSIII* (*Starch Synthases I, II, III*) *SBEI*, *SBEIIa*, *SBEIIb* (*starch branching enzymes I, IIa IIb*), *PUL* (*Pullulanase*) and *ISAI* (*Isoamylase I*) were used as described by Sestili *et al.* (2010). The primers for *CSLF6* (*Cellulose Like Synthase 6*) and Ta2526 were reported in Nemeth *et al.* (2010). Primers specific for *SSIV*, small and large subunits of ADP-glucose pyrophosphorylase (*AGPase*) were described in Kang *et al.* (2013).

Determination of amylose content, total starch, resistant starch, starch damage, β-glucan and water extractable/total arabinoxylan content (WE-AX, TOT-AX)

Amylose content was determined by an iodometric assay on starch granules as reported in Sestili *et al.* (2010). Six technical replicates have been performed for all analyses. A standard curve was obtained using mixtures of potato amylose (Sigma Aldrich) and amylopectin isolated from waxy durum wheat.

Total starch, resistant starch, starch damage and β-glucan content was determined on whole flour or semolina by using the Megazymes Total Starch, Resistant Starch, Starch Damage and β-glucan Assay Kits, respectively (Megazyme International Ireland Ltd., Wicklow, Ireland). WE-AX and TOT-AX were determined by a colorimetric method as described in Finnie *et al.* (2006).

Three technical replicates for each sample were performed. Statistical analysis was performed using one-way ANOVA followed by Tukey t-tests using the R Software.

Protein content

Wheat grains were milled using Cyclotec 1093 sample mill (Foss Tecator, Höganäs, Sweden) to obtain whole flours. Protein content on the ground samples (3.5 g) was determined by a Foss NIRSystem 6500 monochromator (Foss NIRSystems INC., Silver Spring, MD) equipped with a sample transport module and a small ring cup, using calibrations developed at the Società Produttori Sementi Spa (Bologna, Italy), based on NIR guidelines for prediction model development (AACC Method 39-00). Reflectance spectra were taken from 400 to 2500 nm region and recorded as log(1/R) at 2 nm intervals. The WinISI II spectral analytical software (InfraSoft International, Port Matilda, PS) was utilized to

collect and evaluate quantitatively spectra in the analytical procedures. The values of protein content were calculated directly from the system. Each sample was analyzed twice.

Pasting properties by Rapid Visco Analyzer

Starch paste viscosity was measured by the Rapid Visco Analyzer (RVA series 4, Newport Scientific, Sydney, Australia) as described in Sestili *et al.* (2010).

Single Kernel Characterization System

Grain hardness has been determined by measuring crush force, using the Perten Single Kernel Characterization System (SKCS 4100). The SKCS crushes single kernels between a narrowing crescent-shaped gap and toothed rotor to obtain crush-response profiles and conductivity measurements. From 300 kernel samples, these data are algorithmically integrated to provide kernel hardness, weight, diameter, and percent moisture.

Rheological tests

Wheat seeds were cleaned, conditioned to a water content of 16% and left moistened overnight. Semolina samples were obtained by a milling pilot plant (Buhler MLU 202) with three breaking and three sizing passages. Alveographic evaluation was performed on semolina according to the standard method of Chopin alveograph (Chopin, Cedex France) according to standard method UNI 10453. Farinographic tests were carried out by a Brabender apparatus (C.W. Brabender, Duisburg, Germany) according to the standard procedure (ICC 115/1).

Results

Starch properties: amylose content (AC), resistant starch (RS) and pasting profile

Amylose content was determined for the set of mutant lines and values reported as average of the two years of cultivation (Table 1). Data analysis showed an increase in am-

ylose with values ranging from 37% to 46.2% compared with the 30.9% of the control variety Svevo, with large variability among the sister lines. Resistant starch, measured on whole flour, was increased in all the *SSIIa null* wheat lines with values ranging from 2.4% to 3.9% compared with the 0.4% obtained in Svevo.

All the *SSIIa null* durum wheat lines showed a flattened pasting profile, as measured by RVA, characterized by drastically lowered values of all the main parameters (peak viscosity, breakdown, setback and final viscosity) compared to Svevo (Fig. 1). As expected, the viscosity values for the single lines varied in the two years, due to the impact of environmental factors (Supplemental Table 1); nonetheless the trend of the pasting profiles among the mutant lines was conserved. Comparison of viscosity curves led to the identification of four lines (H834, H782, H841, and H788) showing the highest values of peak viscosity (PV) and final viscosity (FV) in both the two years and one line (H838) with the lowest values of PV and FV (Fig. 1, Supplemental Table 1).

Thousand grain weight, total starch, β -glucan and protein content

The hectoliter weight (HW) and 1,000 grain weight (TGW) were reduced at a variable extent (from 10% to 20%) in the set of the fourteen mutant lines compared to Svevo. The reduction in yield parameters was associated with a reduction in total starch content (TS), estimated between 25%–45% reduction. Comparison of the data set revealed the presence of three lines (H782, H834, H788) presenting the lowest loss in total starch among all the mutant lines and one line, namely H838, resulting the worst for its starch content (Table 1).

The β -glucan content was significantly higher in all the *SSIIa null* lines and ranged from 0.28% to 0.5% against the 0.13% of Svevo (Table 1). Notably β -glucan content was more increased in the line H838, that showed the lowest TS content; whereas it was less increased in the set of three best lines described above. A similar behavior was observed for

Table 1. Evaluation of seed and starch related traits of the fourteen *SSIIa null* durum wheat lines

Name	Amylose content (%)	Resistant starch (g/100 g)	Total starch (%)	HW (Kg/hl)	1000 grain weight (g)	Protein content (g/100 g)	β -glucan content (g/100 g)
H742	42.4 $\pm$ 3.2 ^{bc}	2.7 $\pm$ 0.6 ^{bc}	39.9 $\pm$ 2.3 ^b	66.3 $\pm$ 3.2 ^{bd}	21.6 $\pm$ 1.2 ^c	16.4 $\pm$ 0.4 ^d	0.39 $\pm$ 0.21 ^b
H748	44.5 $\pm$ 1.5 ^c	2.8 $\pm$ 0.2 ^{bc}	40.1 $\pm$ 0.9 ^b	62.3 $\pm$ 2.1 ^{bde}	20.5 $\pm$ 4.3 ^{bc}	17.0 $\pm$ 0.5 ^{bd}	0.34 $\pm$ 0.12 ^c
H758	41.2 $\pm$ 2.1 ^b	3.0 $\pm$ 0.2 ^b	35.3 $\pm$ 1.3 ^c	61.7 $\pm$ 1.6 ^{de}	20.2 $\pm$ 4.6 ^{bc}	17.8 $\pm$ 0.4 ^b	0.37 $\pm$ 0.11 ^b
H759	44.1 $\pm$ 1.4 ^c	3.9 $\pm$ 0.1 ^d	37.7 $\pm$ 3.9 ^{bc}	59.0 $\pm$ 1.8 ^c	22.7 $\pm$ 2.3 ^{bc}	16.9 $\pm$ 0.8 ^{bd}	0.38 $\pm$ 0.21 ^b
H770	46.2 $\pm$ 1.3 ^c	3.0 $\pm$ 0.1 ^b	36.8 $\pm$ 1.9 ^{bc}	63.0 $\pm$ 2.0 ^{bd}	24.1 $\pm$ 1.5 ^b	17.5 $\pm$ 0.4 ^d	0.33 $\pm$ 0.05 ^c
H782	40.1 $\pm$ 2.4 ^b	2.8 $\pm$ 0.1 ^{bc}	41.1 $\pm$ 1.1 ^b	66.1 $\pm$ 2.5 ^{bd}	23.3 $\pm$ 1.8 ^{bc}	16.6 $\pm$ 0.8 ^d	0.31 $\pm$ 0.05 ^c
H788	37.3 $\pm$ 3.3 ^b	2.8 $\pm$ 0.2 ^{bc}	40.9 $\pm$ 2.0 ^b	70.3 $\pm$ 1.2 ^c	24.9 $\pm$ 1.6 ^b	18.3 $\pm$ 0.2 ^b	0.34 $\pm$ 0.08 ^c
H834	40.1 $\pm$ 1.5 ^b	2.4 $\pm$ 0.2 ^c	43.5 $\pm$ 3.2 ^b	65.8 $\pm$ 1.2 ^b	23.5 $\pm$ 1.4 ^{bc}	18.0 $\pm$ 1.2 ^b	0.28 $\pm$ 0.06 ^c
H835	37.0 $\pm$ 2.1 ^b	3.2 $\pm$ 0.2 ^b	39.4 $\pm$ 2.1 ^b	67.6 $\pm$ 1.5 ^b	25.8 $\pm$ 0.6 ^b	18.1 $\pm$ 0.6 ^b	0.44 $\pm$ 0.02 ^{bc}
H836	39.8 $\pm$ 3.1 ^b	3.1 $\pm$ 0.1 ^b	39.3 $\pm$ 1.7 ^b	63.7 $\pm$ 2.3 ^{bd}	22.8 $\pm$ 0.8 ^{bc}	18.2 $\pm$ 1.3 ^b	0.36 $\pm$ 0.21 ^c
H838	40.8 $\pm$ 2.8 ^b	3.1 $\pm$ 0.5 ^{bc}	31.3 $\pm$ 2.7 ^c	62.6 $\pm$ 1.1 ^d	23.4 $\pm$ 0.9 ^{bc}	20.2 $\pm$ 0.1 ^c	0.50 $\pm$ 0.03 ^d
H841	42.3 $\pm$ 2.1 ^{bc}	3.2 $\pm$ 0.2 ^b	39.2 $\pm$ 0.7 ^b	71.3 $\pm$ 2.1 ^c	21.6 $\pm$ 2.0 ^c	18.1 $\pm$ 0.8 ^b	0.40 $\pm$ 0.12 ^b
H843	40.0 $\pm$ 3.3 ^b	2.8 $\pm$ 0.2 ^{bc}	40.1 $\pm$ 0.9 ^b	67.8 $\pm$ 1.1 ^b	25.0 $\pm$ 1.9 ^b	17.1 $\pm$ 0.5 ^d	0.42 $\pm$ 0.05 ^b
H848	37.8 $\pm$ 2.2 ^b	2.9 $\pm$ 0.5 ^{bc}	38.5 $\pm$ 3.2 ^b	66.9 $\pm$ 1.2 ^b	25.4 $\pm$ 1.1 ^b	18.3 $\pm$ 0.4 ^b	0.39 $\pm$ 0.03 ^b
Svevo	30.9 $\pm$ 1.2 ^a	0.4 $\pm$ 0.1 ^a	57.2 $\pm$ 2.3 ^a	78.7 $\pm$ 2.1 ^a	37.6 $\pm$ 3.9 ^a	14.5 $\pm$ 1.4 ^a	0.13 $\pm$ 0.04 ^a

Averaged values calculated on two years of cultivation. Different letters indicate significant differences between means for $P < 0.05$.

Fig. 1. Pasting profile of the set of 14 mutant families as measured by RVA. In the figure, Svevo and the mutant lines with the highest (H834, H782, H841 and H788) and the lowest (H838) viscosity curves are indicated. Viscosity (RVU).

protein content (**Table 1**): this increase is likely to be associated with the reduction in TS.

Seed hardness

Seed hardness was determined by SKCS methodology for a set of two *SSIIa* mutant lines (H795 and H806) replicated for two years; data comparison with the control line Svevo did not highlight any significant difference (data not shown).

Correlation among traits

Comparison of amylose content and thousand grain weight data highlighted a significant negative correlation ($r^2 = -0.75$) between the two parameters. A negative correlation has also been detected between amylose and total starch content ($r^2 = -0.815$).

As expected data analysis highlighted a good positive correlation between resistant starch ($r^2 = 0.52$) and amylose content; whereas an inverse correlation of RS was found with TS ($r^2 = -0.77$) and TGW ($r^2 = -0.68$). β -glucans were found to be positively correlated with RS ($r^2 = 0.64$) and at minor extent with amylose content ($r^2 = 0.25$); a negative correlation was found with thousand grain weight ($r^2 = -0.41$) and total starch ($r^2 = -0.77$). A similar profile was found for total protein content: it was correlated positively with RS ($r^2 = 0.39$) but not with AC ($r^2 = 0.06$), and negatively with TS ($r^2 = -0.62$), TGW ($r^2 = -0.28$).

Fig. 2. Relative expression of genes coding for starch and β -glucan biosynthetic enzymes. The analysis were performed on the cultivar Svevo and the *SSIIa null* genotype. Dotted horizontal line at 1 level represents the value of control Svevo.

Rheological evaluation of *SSIIa null* wheat semolina

Semolinas of the two lines H795 and H806, grown in two different years, showed high values of tenacity (P), low values of extensibility (as indicated by the higher P/L ratio), and dough strength (W) compared to the control sample (cultivar Svevo) (**Table 2**, **Fig. 3**). Concerning the farinographic curves, high amylose samples showed higher water absorption, dough development time and degree of softening compared to the cv Svevo; differently, dough stability was significantly lower.

Determination of arabinoxylans and β -glucans in semolina of *SSIIa null* mutant lines

The same lines used for rheological tests were analyzed for arabinoxylan and β -glucan content. TOT-AX and WE-AX content was highly increased in the semolina of *SSIIa null* lines, being 1.5–1.9 and 1.4–1.7 fold higher, respectively (**Table 3**). Also β -glucans were found between 2.7 and 3.4 fold higher in the two lines. Both for arabinoxylans and β -glucans it was found a major increase in the line H806. This result was confirmed in the two years of cultivation.

A strong increase of starch damage was observed for the two lines with higher amylose content compared to Svevo (**Table 3**).

Expression profiles of starch and β -glucans related genes

In order to gain new insights on the molecular mechanisms governing structural changes identified in *SSIIa null* genotypes, a set of genes coding key enzymes involved in starch and β -glucan biosynthesis were investigated at

Table 2. Rheological properties of *SSIIa null* durum wheat semolina

Samples	Alveograph				Farinograph					
	P	L	W	P/L	Water absorption %	Consistency (U.F.)	Dough development (min.)	Dough stability (min.)	Softening degree 10 min. (U.F.)	Softening degree 12 min. (U.F.)
Svevo	120 $\pm$ 6	79 $\pm$ 5	291 $\pm$ 28	2 $\pm$ 0	60	495	4	6	39	68
H795	218 $\pm$ 6	16 $\pm$ 1	159 $\pm$ 10	14 $\pm$ 0	76	528	5	3	77	132
H806	168 $\pm$ 14	10 $\pm$ 1	84 $\pm$ 16	17 $\pm$ 1	83	524	8	4	12	524

Fig. 3. Alveograph (A) and Farinograph (B) tests. The analyses were carried out on semolina of Svevo, H795 and H806 lines.

Table 3. Determination of RS, TOT-AXs, WE-AXs, β -glucans and starch damage for *SSIIa null* semolina

Semolina	Resistant starch	TOT-AXs	WE-AXs	β -glucan	Starch damage
Svevo	1.1 ± 0.2	2.3 ± 0.2	0.59 ± 0.04	0.22 ± 0.04	4.95 ± 0.04
H795	3.1 ± 0.8	3.4 ± 0.3	0.83 ± 0.05	0.60 ± 0.04	6.77 ± 0.03
H806	4.1 ± 0.5	4.2 ± 0.5	1.01 ± 0.12	0.75 ± 0.03	7.19 ± 0.03

All values are expressed in percentages (g/100 g of semolina).

expression level. Analysis was performed on RNA isolated from immature seeds (21 day post anthesis) harvested from the mutant and control lines.

SSIIa genes were confirmed to be down-regulated in the *SSIIa null* mutant; on the contrary all the other major starch synthase genes (*SSI* and *SSIII*), involved in amylopectin synthesis, were found deeply up-regulated, roughly three fold in the double *null* mutant (Fig. 2). Also *GBSSI* (Granule Bound Starch Synthase) expression was 2.5 fold increased compared to the wild type. *SSIV* gene was found to be less up-regulated than other SSs in the *SSIIa null* genotype in agreement with the role of this enzyme, majorly involved in the synthesis of primary starch in the leaf tissue. Differently the gene expression of starch branching enzymes was poorly affected in the wheat mutant line, except for *SBEI* gene that was found slightly up-regulated (1.5 fold). No appreciable differences were revealed for the genes coding debranching enzymes (*LDA* and *ISA*); lastly, a significant down-regulation of the *AGPase* small subunit gene was detected.

Cellulose Synthase-Like F6 (*CSLF6*) is known to be one

of the major enzyme regulating β -glucans synthesis in wheat (Nemeth *et al.* 2010). Interestingly, our data indicated a strong increase of the expression of the *CSLF6* gene in the *SSIIa null* line (6.4 fold) in respect to the control line.

Discussion

Analysis of the set of fourteen *SSIIa null* lines

In classical plant breeding the introgression of a desired trait can often be associated with pleiotropic effects some of which may have adverse effects on the overall plant performances. Crossing of the mutant plant with a high performing variety represents a valuable strategy to combine the new trait and the maintenance of good agronomic performances (Howard *et al.* 2014). In this work we report the characterization of major seed traits in a durum wheat genotype derived from the introgression of *SSIIa null* character in the elite cultivar Svevo. A large set of *SSIIa null* lines (144) were produced by four backcrossing cycles with the parental line Svevo, then characterized for a set of major traits such as grain yield, seed color, amylose content and viscosity properties (data not shown). A significant variability among the wheat lines was detected: from the observation of these data, a small set of families were selected and characterized either to better elucidate the relationship between *SSIIa null* alleles and pleiotropic effects on grain traits or to produce an improved *SSIIa null* durum wheat. In particular, lines with the best and worst values for grain yield were selected. The increased level of amylose amount in our mutant lines confirmed that these materials are of

interest for their unique nutritional characteristics. Interestingly a wide variability has been reported in the response of different cereals to the absence of *SSIIa* enzyme: in diploid barley amylose content was 70% in the *SSIIa null* genotype compared to the 25% of the control line (Morell *et al.* 2003, Sparla *et al.* 2014); in bread wheat the increase in amylose was modest resulting 37% in the mutant compared to the 29% of the wild type (Yamamori *et al.* 2000). We previously found that in durum wheat the increase in amylose content was an intermediate value between that of barley and bread wheat; this data was recently confirmed by Hogg *et al.* (2013) that produced *SSIIa null* durum wheat.

Amylose content was found to vary from 37.0% to 46.2% among the set of fourteen *SSIIa null* sister lines. This variation is likely to be attributable to the different genetic backgrounds possessed by the different lines. This observation is in agreement with the hypothesis of the existence of minor factors regulating amylose content in cereals. At this purpose Wu *et al.* (2009) suggested that the action of major loci regulating amylose content (as *waxy1*, *sugary-2*, *sugary-1* and *dull*) is quantitatively conditioned by minor modifier genes in amylomaize VII corn. Mohammadkhani *et al.* (1999) reported similar considerations for einkorn and emmer wheats and rye. As expected all the fourteen mutant lines showed a RVA profile typical of the high amylose starches in which all main parameters had strongly lowered values compared to the control (Yamamori *et al.* 2006).

Our data confirmed the existence of a positive correlation between amylose and resistant starch contents in the durum wheat mutant lines; RS values were comparable with those for *SSIIa-null* genotype of bread wheat and slightly lower in respect to the *SBEIIa null* durum wheat (Sestili *et al.* 2015, Slade *et al.* 2012). Hazard *et al.* (2012) found a minor increase of RS in the *SBEIIa* mutant of the durum wheat Kronos (less than 1%) likely due to the different genetic background of the cultivar or due to the type of mutation affecting *SBEIIa* gene expression. In bread wheat co-suppression of both *Sbella* and *Sbellb* resulted with higher values of RS (Regina *et al.* 2015).

Other parameters known to be affected in *SSIIa null* genotypes are thousand grain weight, total starch and protein content as previously reported for bread wheat and barley (Konik-Rose *et al.* 2007, Morell *et al.* 2003, Yamamori *et al.* 2000). Our data confirmed a much more drastic effect on total starch and seed weight in durum compared to bread wheat as already highlighted recently by Hogg *et al.* (2013).

The increase of protein content, observed in *SSIIa null* lines, is in agreement with the values measured by Konik-Rose *et al.* (2007) in the bread wheat *SSIIa* mutant. They supposed that the apparent increase in protein content did not represent more protein synthesis per grain, but it reflected the strong decrease in starch content.

In all fourteen *SSIIa null* lines β -glucan content was strongly increased compared to Svevo. β -glucans are present in the starchy endosperm cell wall representing 20% of the total. An increase in β -glucan content was previously

reported both in waxy and high amylose genotypes of barley (Clarke *et al.* 2008, Howard *et al.* 2014, Xue *et al.* 1997). Similarly, β -glucan has been found increased in the waxy genotypes of bread wheat (Ahuja *et al.* 2013, Yasui *et al.* 1999). The increase in β -glucans originates by common substrates or enzymes between starch and β -glucan synthesis; here we provide the evidence that also in high amylose durum wheat, β -glucans content is significantly increased. The lack of *SSIIa* is associated with a decrease in amylopectin synthesis and total starch content, confirmed by the *wrinkled/shrunken* seed phenotype observed in our *SSIIa null* durum wheat lines; so it can be postulated that the ADP-glucose not processed by *SSIIa* enzyme becomes available to the other biosynthetic pathways such as that of β -glucans. A similar model was recently proposed by Islamovic *et al.* (2013) who, studying the genetic dissection of β -glucan and amylose content in barley, suggested an intricate co-expression network that regulates polysaccharide content in the kernel.

Zhang *et al.* (2012) studied carbon reallocation to other carbohydrates end products in a rice waxy mutant, reporting that only the amylopectin content increased significantly; while β -glucans, other sugars and also total starch remained almost unchanged. In contrast, Howard *et al.* (2014) recently reported an increase of β -glucans of 40% in a waxy barley in respect to the control; the same authors found an increase of the β -glucans/total starch ratio in a *SSIIa null* defective barley.

qRT-PCR experiment showed that the gene coding CSLF6 the major enzyme synthesizing β -glucans in wheat (Nemeth *et al.* 2010), was deeply up-regulated in the *SSIIa null* mutant compared to Svevo. This result correlates with the increase in β -glucan amount observed in whole flours of the mutant lines.

Various reports have investigated the relationship between amylose content and seed hardness in cereals. In maize it was proposed that hard endosperms were richer in amylose compared to soft ones (Dombrink-Kurtsman and Knutson 1997). Similarly, Clarke *et al.* (2008) reported an increase in seed hardness in a high amylose *SSIIa null* mutant of barley compared to the control line. In the material here analyzed the correlation between seed hardness and amylose content has not been observed.

Semolina characterization

The preliminary evaluation of the rheological characteristics of two elevated amylose lines highlighted a large difference in both alveographic and farinographic values and, in addition, higher water absorption compared to Svevo. This can be attributed to the increase in fiber components, such as arabinoxylans and β -glucans, and a higher starch damage (Turner *et al.* 2008).

Notably the semolina of the two lines H795 and H806 presents a significant increase in total arabinoxylans content. Arabinoxylans, together with β -glucans, represent the major dietary fiber in wheat grain, accounting for the 70%

of the total cell wall polysaccharides, and are associated to a number of health benefits.

SSIIa null semolina is a raw material of particular interest for its potential health benefits as it presents an increase of different classes of dietary fibers, namely, resistant starch, arabinoxylans and β -glucans (Wood 2007). Hogg *et al.* (2015) recently confirmed an increase in total fiber also in the pasta made by a *SSIIa null* durum wheat line.

Expression profiles of starch related genes

The expression of a set of major genes directly involved in starch biosynthesis was investigated in a *SSIIa null* genotype. Among major classes of starch biosynthetic enzymes we found a significant increase in the expression of starch synthases; no detectable changes were found in the genes coding starch branching enzymes. The increase in the expression of SSs is likely to be due to a mechanism of compensation due to the absence of SSIIa. Interestingly GBSSI gene was also found up-regulated suggesting an attempt of the cell to metabolize glucose accumulated in the amylopectin defective mutant. Moreover the analysis showed a decrease of the transcript level of AGPase, the enzyme synthesizing ADP-glucose; this seems to be in agreement with the reduction in total starch observed in the *SSIIa null* sister lines. Our data are not in agreement with those of Hogg *et al.* (2013) who found an increase in expression of the genes coding a large subunit of AGPase and starch branching enzymes. These differences could be due to the different genetic backgrounds of the two *SSIIa null* durum wheats.

A further pleiotropic effect associated with the absence of the SSIIa proteins is the abolishment of the presence of the proteins Sgp-2 (SBEII) and Sgp-3 (SSI) within the starch granules (Kosar-Hashemi *et al.* 2007, Lafiandra *et al.* 2010). However our qRT-PCR experiment highlighted that these genes were not silenced: in particular no detectable changes were found in starch branching enzymes, whereas the SSI gene was even up-regulated three fold in the mutant compared to Svevo. These data support the hypothesis that the loss of the two proteins (Sgp-2 and Sgp-3) in the starch granule corresponds to their increase in the protein soluble fraction, due to the absence of the “starch granule protein complex” in the *SSIIa null* genotype (Luo *et al.* 2015).

In conclusion durum wheat collection has been enriched of new lines with interesting nutritional characteristics through the implementation of a classic breeding strategy applied to reduce undesired effects associated to *SSIIa null* mutant genotypes. Analysis of our data led to the identification of a set of lines showing acceptable total starch and grain yield although possessing values of amylose content and resistant starch typical of *SSIIa null* genotype.

Preliminary rheological data indicates that processing of these material (e.g. milling) need to be optimized using conditions specific for its characteristics and composition.

In addition, a preliminary characterization of semolina highlighted an altered composition of the non starch polysaccharides fraction that constitutes the main dietary fiber in

wheat grains.

Worthy of note these materials can be defined “fiber enriched” for the increase in resistant starch, β -glucans and arabinoxylans and can provide further health benefits acting synergistically with resistant starch.

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