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H. Funatsuki · H. Kurosaki · T. Murakami
S. Matsuba · K. Kawaguchi · S. Yumoto · Y. Sato

Deficiency of a cytosolic ascorbate peroxidase associated with chilling tolerance in soybean

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Abstract We investigated the isozyme profiles of antioxidant enzymes in cultivars and lines with different seed productivity in cool climate conditions as a step towards understanding the physiological and genetical mechanisms underlying chilling tolerance in soybean. While no difference in superoxide dismutase, or catalase isozyme profiles was observed among the cultivars and lines tested, we found polymorphism in the ascorbate peroxidase isozyme profile; there were two types, with or without a cytosolic isoform (APX1). The cultivars and lines lacking APX1 proved more tolerant to chilling temperatures, as evaluated by yielding ability. The genotype-dependent deficiency of APX1 was consistent in plants and tissues under various oxidative stress conditions including the exposure to low-temperatures. In addition, the genetic analysis of progeny derived from crossing between cultivars differing in the isozyme profile indicated that the APX1 deficiency is controlled by a single recessive gene (*apx1*), and is inherited independently of the genes that have previously been identified for their association with chilling tolerance. Molecular and linkage analyses suggested that the variant gene of the APX1-absent genotype coding for a cytosolic APX, which contained a single nucleotide substitution and a single nucleotide deletion in the coding region, is responsible for the genotype-dependent deficiency of APX1. The association of APX1 deficiency with chilling tolerance is discussed in detail.

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H. Funatsuki (✉) · T. Murakami · S. Matsuba · K. Kawaguchi
Y. Sato
National Agricultural Research Center for Hokkaido Region
(NARCH), Hitsujigaoka, Sapporo, Hokkaido 062-8555, Japan
e-mail: funazki@affrc.go.jp
Tel.: ++81-11-857-9312, Fax: ++81-11-859-2178

H. Kurosaki · S. Yumoto
Tokachi Agricultural Experiment Station (TAES), Shinnsei,
Memuro, Hokkaido 082-0071, Japan

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Introduction

Many of the world's important crops are now widely distributed beyond their original zones of natural selection and hence their yields are constrained by what have been termed thermal thresholds for optimal growth (Greaves 1996). The cultivation of soybean, which prefers a warm climate, has continued its expansion into cool areas including Canada, Sweden, Siberia and Hokkaido, the northernmost part of Japan, where soybean plants sometimes suffer from chilling injury. It is known that there are considerable genetic variations in the chilling tolerance of soybean (Raper and Kramer 1987). Some cultivars from Hokkaido and the islands of the Okhotsk Sea, for instance, have a somewhat lower minimum temperature for good flowering and pod set than 17 °C, which is required for standard cultivars (Raper and Kramer 1987). The understanding of physiological and genetical mechanisms of such chilling-tolerant cultivars is of major interest for both soybean breeders in cool areas and plant scientists investigating the regulatory mechanisms of plants to low-temperature stress.

Soybean breeders and researchers have identified several genes associated with chilling tolerance. They started with seeking genes found commonly in chilling-tolerant cultivars, based on the visual observation and the comparison of the response of genotypes to variable temperatures in different locations and years (Sanbuichi 1979; Yumoto and Tsuchiya 1991; Morrison et al. 1994). They subsequently identified the genes associated with chilling tolerance by comparing near-isogenic lines or recombinant inbred lines with different alleles at the locus of interest (Sanbuichi 1979; Takahashi and Asanuma 1996; Morrison et al. 1997). The lines with the genes, *p1* (pubescent surface) and *T* (tawny pubescence), were proved to be more tolerant to low temperature at

the level of seed yielding ability, compared to those with the other alleles. However, the possibility of finding novel genes conferring chilling tolerance through this approach is limited because the candidate genes need to influence visible traits.

In the last decade, physiological and molecular-biological studies have made great advance in understanding the mechanism underlying chilling tolerance. Among many factors affecting chilling tolerance, the capacity of scavenging reactive oxygen species (ROS), such as hydrogen peroxide and superoxide, has been demonstrated to play an important role in chilling-sensitive plant species such as maize (Prasad et al. 1994; Hodges et al. 1997; Pinhero et al. 1997) and rice (Saruyama and Tanida 1995; Sato et al. 2001), since chilling caused elevated levels of ROS (Prasad et al. 1994). Primarily, the importance of the scavenging capacity through the ascorbate-glutathione cycle was stressed on its role in chloroplasts (Foyer et al. 1994; Asada 1999). However, the scavenging capacity was also reported to play a key role in other subcellular compartments, especially in stress conditions (Prasad et al. 1994; Mittler and Zilinskas 1994; Sato et al. 2001).

Since the tolerance to chilling stress was enhanced in transgenic plants that expressed exogenous genes encoding ROS-scavenging enzymes (Gupta et al. 1993; Roxas et al. 1997; Van Breusegem et al. 1999a, b), the intraspecific variation in isozyme profiles may cause the differences in chilling tolerance. Since activity staining, which is a valuable method for detection of polymorphic isozyme profiles, is available for some ROS-scavenging enzymes (Beachamp and Fridovich 1971; Woodbury et al. 1971; Mittler and Zilinkas 1993), it is possible to explore intraspecific variations in profiles of these isozymes. From a plant breeders' point of view, a polymorphic isozyme profile can be used in marker-assisted selection in plant breeding programs (e.g. Konishi et al. 1997).

In the present study, we examined isozymes of the major ROS-scavenging enzymes, superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and ascorbate peroxidase (APX, EC 1.11.1.11) in soybean genotypes differing in chilling tolerance. We report the genetic association between deficiency of an APX isozyme and chilling tolerance, and the genetic and molecular basis of the APX deficiency.

Materials and methods

Plant material

Twenty cultivars and lines of soybean developed in the TAES were used for the present study (Table 1). Crossing for progeny tests was performed in the combinations between Kitamusume and Hokkaihadaka, and between Toyomusume and Hayahikari. The hybridity of F_1 plants was confirmed by the dominant trait that the male parent in each cross combination possessed, a glabrous surface for Hokkaihadaka and tawny pubescence for Hayahikari. In the latter combination, the F_4 generation was true-bred by the single-seed descent method.

Table 1 Degrees of chilling tolerance and APX1 expression of 20 soybean cultivars and strains

Cultivar/strain	Pubescence	CTI[a]			APX1[b]
		1997	1998	Mean	
Tokei 834	Gray (*p1*, *t*)	91	77	84	–
Tokei 738	Tawny (*p1*, *T*)	80	78	79	–
Hayahikari	Tawny (*p1*, *T*)	78	79	78.5	–
Tokei 803	Tawny (*p1*, *T*)	86	69	77.5	–
Tokei 806	Gray (*p1*, t)	81	62	71.5	–
Toyokomachi	Gray (*p1*, t)	86	52	69	+
Kitamusume	Tawny (*p1*, *T*)	69	63	66	–
Toyohomare	Gray (*p1*, t)	66	61	63.5	–
Tokei 113	Tawny (*p1*, *T*)	69	58	63.5	+
Tokei 867	Gray (*p1*, t)	76	50	63	+
Tokei 793	Gray (*p1*, t)	60	62	61	+
Hokkaihadaka	Glabrous (*P1*)	90	31	60.5	+
Tokei 853	Gray (*p1*, t)	60	57	58.5	+
Tokei 130	Tawny (*p1*, *T*)	65	49	57	+
Tokei 805	Gray (*p1*, t)	57	51	54	+
Toiku 112	Tawny (*p1*, *T*)	53	48	50.5	+
Suzuhime	Gray (*p1*, t)	56	36	46	+
Toyosuzu	Gray (*p1*, t)	53	39	46	+
Tokei 769	Gray (*p1*, t)	56	32	44	+
Toyomusume	Gray (*p1*, *t*)	48	30	39	+

[a] CTI = (Seed yield at a cold location)/(Seed yield at the control location) × 100
[b] +: present, –: absent

Field tests

In 1997 and 1998 field experiments were conducted at two locations. One was the research field of the TAES in Memuro, Hokkaido, which was used for the control field. The other was the test field for chilling tolerance in Kamishihoro, Hokkaido, where the mean temperature is 1–2 °C lower than that in Memuro during the growing season of soybean (from June to October). The field in Kamishihoro has been successfully used to select chilling-tolerant lines since the 1960s (Sanbuichi 1979). A rate of 16.7 plants/m^2 was used in a plot. A plot was composed of two rows 3-m long, with spacing of 60 cm during 1997, while the plot area was doubled in 1998 by adding extra two rows. Replication was not added in either of the fields. Plants were harvested at maturity. Chilling tolerance index (CTI) was calculated for each cultivar and line according to Sanbuichi (1979) as follows:

$$\text{CTI} = (\text{Seed yield in Kamishihoro})/(\text{Seed yield in Memuro}) \times 100$$

Growth conditions and stress treatments

For isozyme analyses, plants were grown in growth chambers. Seeds were sown in pots containing soil or vermiculite and grown under the regime of 15 h light (approximately 300 μmol m^{-2} s^{-1} at canopy height) at 22 °C and 9 h dark at 17 °C. Half-strength Hoagland solution was added daily. Plants were sampled when the second trifoliate leaves were fully expanded, and were maintained until the appropriate stages for harvest of reproductive organs.

For testing cytosolic APX expression in response to various stresses, plants at the developmental stage as described above, or leaf discs (approximately 7 mm in diameter) were exposed to several treatments as follows. Low- and high-temperature stress: plants were grown at 4 °C/4 °C, 15 °C/10 °C, 30 °C/25 °C, and 42 °C/42 °C (day/night) for 1 day. In the milder conditions (15 °C/10 °C, 30 °C/25 °C), treatment periods were extended up to 1 week. For H_2O_2 and methyl viologen treatments, leaf discs were incubated on the surface of distilled water containing H_2O_2

or methyl viologen for 12 h in darkness, followed by exposure to light (approximately 150 μmol m^{-2} s^{-1}) for 8 h. The concentrations were 1 mM, 10 mM of H_2O_2, and 1 μM, 10 μM of methyl viologen. For high intensity light treatment, leaves were irradiated with approximately 2000 μmol m^{-2} s^{-1} of light at 30 °C for leaf temperature of 1 h. For UV treatment, leaf discs were exposed to 100 mJ of UV and incubated for 1 day on the surface of distilled water. Drought treatment, plants were grown until the relative fresh weight decreased by 20% after watering had been stopped. For salt treatment, 120 mM of NaCl was supplied for 4 days. When leaves were harvested, visibly apparent damage was observed for all treatments except for high intensity light stress and treatments with H_2O_2 and methyl viologen at the lower concentrations (1 mM and 10^{-6} M, respectively).

Gel assay

Cell-free extracts were analyzed on nondenaturing gels, according to Anderson et al. (1995) and Caldwell et al. (1998), with minor modifications. Plant samples were harvested and 1 g of tissue was frozen in liquid nitrogen and ground into powder, which was suspended in 2.5 ml of 50 mM potassium phosphate, pH 7.8, containing 1 mM of EDTA, 0.25 mM of PMSF and 125 mg of polyvinylpolypyrrolidone with a mortar and pestle at 4 °C. In the experiments where leaf discs were used, buffer volume was reduced according to their fresh weight. For analysis of APX and CAT, the extraction buffer also contained 2 mM of ascorbic acid and 10 mM of DTT, respectively. The insoluble fraction was removed by centrifugation at 14,400 g for 15 min at 4 °C. Protein content was determined according to Bradford (1976).

Isozymes of SOD were separated on 10% nondenaturing polyacrylamide gels, which were stained after electrophoresis, according to Beachamp and Fridovich (1971). Isozymes of CAT were separated on 7% nondenaturing polyacrylamide gels, which were stained as described by Anderson et al. (1995). Isozymes of APX were separated on 10% non-denaturing polyacrylamide gels and stained according to Caldwell et al. (1998). Electrophoresis for all assays was carried out in an electrophoresis cell (Protean Mini III, BioRad), which was soaked in iced water to maintain the buffer temperature below 4 °C.

APX assay

The activity of total APX was determined spectrophotometrically as described by Saruyama and Tanida (1995), with a minor modification that sodium azide was omitted in the reaction mixture.

Western-blot analysis

Electrophoresis was performed as described in the Gel assay. Proteins were transferred onto a PVDF membrane (Hybond P, Amersham Pharmacia Biotech). Washing and incubation with primary antibody (cyt-mAb1, Yoshimura et al. 2001) were carried out according to Yoshimura et al. (2001). HRP-conjugated sheep anti-mouse IgG (Amersham Pharmacia Biotech) was used as a secondary antibody. Signals were detected using an ECL-Plus kit (Amersham Pharmacia Biotech).

RT-PCR and real-time PCR

Total RNA was isolated from plant tissues using RNeasy Plant Mini Kits (Qiagen). The isolation of mRNA and the synthesis of cDNA were performed with TaKaRa mRNA isolation and cDNA synthesis kits (TaKaRa). cDNAs were amplified with a high-fidelity PCR enzyme, ProofStart DNA Polymerase (Qiagen) involved 35 cycles of 94 °C for 30 s, 65 °C for 30 s and 72 °C for 1 min. The sequences of two primer sets were designed on the basis of the published sequences (Chatfield and Dalton 1993; Caldwell et al. 1998). In the first primer set, which was to amplify cDNA (*GmAPX1*) including the whole coding region corresponding to *pSOYAP75* (Chatfield and Dalton 1993) and *SOYAPx1* (Caldwell et al. 1998), one primer was homologous to the 5′ end (5′-CTCCATTTCCATTTTCTCTCTCAC-3′), and the second primer, in reverse orientation, was homologous to the 3′ end (5′-ATATTATGGGCACCATGCCTCTT-3′). In the second primer set, which was to amplify cDNA (*GmAPX2*) including the whole coding region corresponding to *SOYAPX2* (Caldwell et al. 1998), one primer was homologous to the 5′ end (5′-CCATTTCCATTTCCTCTCTCTCAG-3′), and the second primer, in reverse orientation, was homologous to the 3′ end (5′-ATATTATGGGCACCGTGCCTCTC-3′). Amplified cDNA fragments were cloned in the TA-cloning vector pGEM-T Easy (Promega) according to the manufacturer's instruction. In each case, reactions were performed three times, and PCR products were sequenced on a LIC-4200L-2 DNA sequencer (Li-cor). Assembly and comparison of DNA sequence data was done using DNASIS software (Hitachi Software Engineering). For real-time PCR, cDNA samples prepared from three independent isolations of total RNA of each cultivar were used as templates. The quantification of transcripts by real-time PCR was carried out using a LightCycler with the FastStart DNA Master SYBR Green I Kit (Roche Molecular Biochemicals). Standard curves were made with serially diluted plasmids (100 pg–10 fg) containing either of *GmAPX1* or *GmAPX2*.

Northern-blot analysis

Twenty micrograms of total RNA was separated on a 1.2% (w/v) agarose gel containing 16% (v/v) formaldehyde and transferred by capillary blotting to a Hybond N^+ (Amasherm Pharmacia Biotech) hybridization membrane. Hybridization using probes to detect *GmAPX1* and *GmAPX2* transcripts was carried out at 65 °C for 16 h in the Rapid-hyb buffer (Amasherm Pharmacia Biotech) containing 100 μg/ml^{-1} of salmon-sperm DNA. Radiolabeled probes were prepared using the Megaprime labeling system (Amersham Pharmacia Biotech). Following hybridization, membranes were washed twice in 2 × SSC and 0.1% (w/v) SDS for 20 min at room temperature, once in 1 × SSC and 0.1% (w/v) SDS for 20 min at room temperature, and finally twice in 0.1 × SSC and 0.1% (w/v) SDS for 30 min at 65 °C. Signals were detected using autoradiography, and signal intensities were quantified using a Typhoon 8600 variable mode imager and Imagequant software (Amersham Pharmacia Biotech).

PCR with genomic DNA

Genomic DNA was isolated from leaf tissues of Kitamusume, Hokkaihadaka, a F_1 hybrid of these two cultivars and F_2 plants using the PrepManUltra kit (Perkin-Elmer). Two reactions were performed for one sample. In both reactions, one primer was homologous to a part of cDNA sequence of *GmAPX1* (5′-AGGACGAAGATGCCTTCTTT-3′). Second primers, in the reverse direction, were designed to specifically amplify fragment of *GmAPX1* gene derived from Kitamusume (5′-AAACGTTGCTTAGGCATCAGCTAC-3′) and from Hokkaihadaka (5′-AAACGTTGCTTAGGCATCAGCTAA-3′), according to Drenkard et al. (2000). The cycling condition started with initial denaturation at 94 °C for 3 min, followed by 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, for 30 cycles. Amplified fragments were separated on 4% agarose gels.

Results

Chilling tolerance among cultivars and lines

Twenty cultivars and lines, which varied in chilling tolerance in preliminary experiments, were grown at the test field for evaluating chilling tolerance in 1997 and

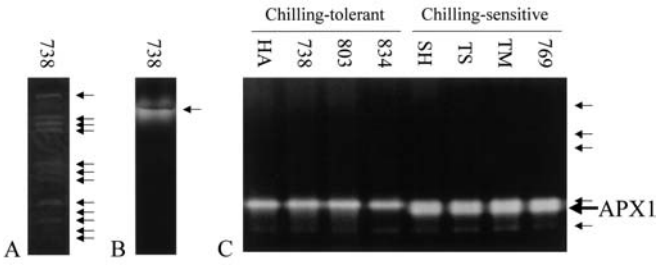

Fig. 1 Zymogram analyses of SOD (**A**), CAT (**B**) and APX (**C**) activities in soybean leaves of four chilling-tolerant and four chilling-sensitive genotypes. Enzyme activities were visualized by the activity staining procedure following separation on non-denaturing polyacrylamide gels. HA, 738, 803, 834, SH, TS, TM and 769 represent Hayahikari, Tokei 738, Tokei 803, Tokei 834, Toyosuzu, Toyomusume and Tokei 769, respectively. *Arrows* represent the positions of the isozymes observed. The *arrow* with APX1 shows the position of the cytosolic APX with higher mobility

1998. The chilling tolerance index (CTI, see Materials and methods) of each cultivar and line was calculated based on the comparison with its performance at the control field (Table 1). In both years there were considerable genotypic variations in CTI. Although a little difference within genotypes in terms of chilling tolerance based on CTI was observed between years, CTI in the 2 years was significantly correlated with each other (correlation coefficient = 0.83, $P < 0.01$). The analysis of variance confirmed the genotypic difference in CTI ($P < 0.01$) as well as the annual fluctuation ($P < 0.001$). Based on the highest and lowest mean CTI over 1997 and 1998, we selected Tokei 803, Tokei 738, Tokei 834 and Hayahikari as tolerant genotypes, and Toyomusume, Suzuhime, Toyosuzu and Tokei 769 as sensitive ones for further analyses of isozymes.

Gel assays of SOD, CAT and APX

We observed 12 SOD isozymes on nondenaturing polyacrylamide gel (Fig. 1A). However, there was no polymorphism in any isozyme among the eight cultivars and lines. In the gel assay for CAT isozyme, only one broad band, which may be composed of more than one isozyme, was detected (Fig. 1B) in all samples. Gels stained for APX activity revealed two prominent and four faint bands (Fig. 1C). The two major bands appeared to correspond to the two isoforms of cytosolic APX (cAPX) reported by Caldwell et al. (1998), and polymorphism was observed in the more mobile one. All four chilling-tolerant cultivar and lines lacked this isozyme, while all chilling-sensitive genotypes had the isozyme. We designated this isozyme as APX1.

Table 2 Comparison of degrees of chilling tolerance and total APX activities between groups differing in the expression of APX1

Phenotype	No. of cultivars and lines	CTI[a]		APX activity[b]
		1997	1998	
APX1$^-$	7	79	69	2.34
APX1$^+$	13	64	46	3.41
t-test		$P < 0.01$	$P < 0.001$	$P < 0.001$

[a] See Materials and methods
[b] μmol ascorbate mg protein^{-1} min^{-1}

Comparison of CTI between APX1-present and -absent genotypes

To examine whether the polymorphism found in the APX isozyme profile is associated with chilling-tolerance, we determined the presence or absence of APX1 in the other 12 cultivars and lines. In addition to the four chilling-tolerant genotypes, Kitamusume, Toyohomare and Tokei 806 proved to lack APX1 (Table 1). The mean CTI of APX1-absent genotypes was significantly higher than that of APX1-present genotypes in both years (Table 2). In addition, only cultivars and lines with tawny pubescence were compared to eliminate the possible distortion of CTI due to the different proportions of that genotype between the two groups. Then the mean CTI of APX1-absent cultivars and lines was still significantly higher than that of APX1-present ones (75 vs 58, $P < 0.05$). Total APX activities also differed between the two genotypes (Table 2).

Stability of the polymorphism of cAPX

To examine whether the genotype-dependent polymorphic profiles of cDNA are conserved in various tissues and under various oxidative stress conditions, we used two varieties, Kitamusume (APX1-absent) and Hokkaihadaka (APX1-present), which are relatively closely related. The isozyme profiles of cAPX were consistent in expanded leaves, developing leaves, stems, roots and nodules of APX1-absent and APX1-present plants (Fig. 2). The same isozyme profiles were also observed in reproductive organs, such as anthers, pods and immature seeds (data not shown). In addition, as it is known that cAPX can be induced or enhanced by oxidative stresses (Mittler and Zilinskas 1992; Kubo et al. 1995; Donahue et al. 1997; Karpinski et al. 1997; Morita et al. 1999; Yoshimura et al. 2000, Sato et al. 2001), we examined whether APX1 is expressed after treatments that are considered to cause oxidative stress. Although various kinds of treatments, such as exposure to high and low temperatures, H_2O_2, methyl viologen, high-intensity light, UV, drought and NaCl, were tested; they neither generated bands with higher mobility in Kitamusume, nor suppressed APX1 in Hokkaihadaka (Fig. 2), except

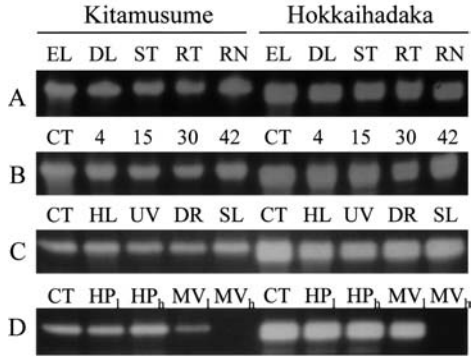

Fig. 2A–D Influences of organs and stress treatments on the expression of cAPX isozymes. Samples were prepared from an APX1-deficient cultivar, Kitamusume, and an APX1-present cultivar, Hokkaihadaka. Soluble proteins were extracted from expanded leaves except for gel **A**, on which extracts from developing leaves (*lanes DL*), stems (*lanes ST*), roots (*lanes RT*) and root nodules (*lanes RN*) were used in addition to expanded leaves (*lanes EL*). On gel **B–D**, *lanes CT* were assigned for the controls, which were grown or cultured at 22 °C/17 °C without any stress treatments. On gel **B**, Effects of various temperatures were examined at 4 °C/4 °C (*lanes 4*), 15 °C/10 °C (*lanes 15*), 30 °C/25 °C (*lane 30*), and 42 °C/42 °C (*lane 42*). On gel **C**, effects of environmental stresses were examined with high-intensity light (*lanes HL*), UV irradiation (*lanes UV*), drought (*lanes UV*), and salt (*lanes SL*). On gel **D**, effects of H_2O_2 were examined at the concentrations of 1 mM (*lanes HP$_l$*) and 10 mM (*lane HP$_h$*), and effects of methylviologen at 1 μM (*lane MV$_l$*) and 10 μM (*lanes MV$_h$*)

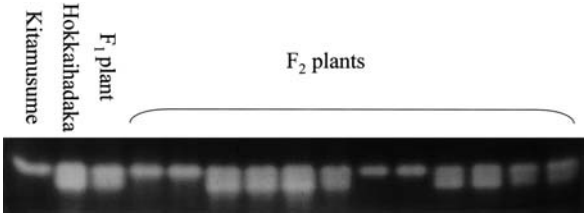

Fig. 3 cAPX isozyme expression in the progeny of the cross between an APX1-absent genotype, Kitamusume, and an APX1-present genotype, Hokkaihadaka. APX activities were visualized by the activity staining procedure after the separation on the non-denaturing polyacrylamide gel

that all isozymes disappeared at the higher concentration of methyl viologen treatment.

Inheritance of the cAPX deficiency

In F_1 hybrids resulting from the cross between Kitamusume and Hokkaihadaka, APX1 was clearly present (Fig. 3). F_2 plants segregated at the expected ratio of 3 APX1-present: 1 APX1-absent, suggesting that the deficiency of APX1 is controlled by a single recessive gene, *apx1* (Fig. 3, Table 3). The segregation of F_4 plants derived from the cross between Toyomusume (APX1-present) and Hayahikari (APX1-absent) developed by the single-seed descent method agreed with the expected ratio of 9 APX1-present: 7 APX1-absent (Table 3). In addition, the APX deficiency appeared to be transmitted independently of the genes, *p1* and *T*, which have previously been reported to associate with chilling tolerance at the seed yield level (Table 3).

Expression and sequence analyses of cAPX

Two cDNAs for cAPX (*SOYAPx1* and *SOYAPx2*) have already been cloned (Caldwell et al. 1998), although it has not been determined which of the cDNAs relates to the cAPX with higher mobility. We therefore analyzed both cDNAs from Kitamusume and Hokkaihadaka. Using primers designed from the published sequences, protein-coding regions of two cDNAs for cAPX, tentatively designated as *GmAPX1* and *GmAPX2*, which corresponded to *SOYAPx1* and *SOYAPx2*, respectively, were amplified by RT-PCR in both cultivars (Fig. 4A, B). Northern-blot analysis, in which the probe used hybridized with both mRNAs derived from the two genes encoding cAPX because of the high degree of sequence homology, revealed no significant difference in the steady state level of total transcripts for cAPX between the cultivars (Figs. 4C, 5A). However, Western-blot analysis with monoclonal antibody against cAPX (cyt-mAb1, Yoshimura et al. 2001) clearly demonstrated that Kitamusume lacked one of the cAPX proteins, lead-

Table 3 Segregation of F_2 and F_4 plants from the crosses between APX1-present and APX1-absent cultivars for APX1 expression and genetic association with other genes associated with chilling tolerance

Generation and parents	Phenotype	APX1$^+$	APX1$^-$	Total	χ^2	Probability
F_2 Kitamusume (*p1*, APX1$^-$) × Hokkaihadaka (*P1*, APX1$^+$)	Non-recombinant	55 (*P1*)	3 (*p1*)	58	0.18 (5:3)[a]	0.5 < *P* < 0.75
	Recombinant	16 (*p1*)	22 (*P1*)	38		
	Total	71	25		0.06 (3:1)[b]	0.75 < *P* < 0.9
F_4 Toyomusume (*t* APX1$^+$) × Hayahikari (*T*, APX1$^-$)	Non-recombinant	25 (*t*)	29 (*T*)	54	0.30 (63:65)[a]	0.5 < *P* < 0.75
	Recombinant	34 (*T*)	16 (*t*)	50		
	Total	59	45		0.01 (9:7)[b]	0.9 < *P*

[a] Segregation ratio that is expected, given the expression of APX1 is inherited independently of *P1*/*p1* or *T*/*t*
[b] Segregation ratio that is expected, given that APX1$^-$ is controlled by a single recessive gene

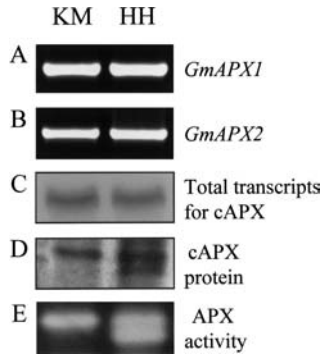

Fig. 4A–E Detection of transcripts, proteins and activities of cAPX isozymes in Kitamusume (*KM*), and Hokkaihadaka (*HH*). *GmAPX1* cDNA (**A**) and *GmAPX2* cDNA (**B**) fragments were amplified by RT-PCR. Northern-blot analysis of *GmAPX1* and *GmAPX2* were performed using *GmAPX1* as a probe (**C**). Western-blot analysis of cAPX proteins were performed using the monoclonal antibody against cAPX (**D**). APX activities were visualized by the activity staining procedure (**E**)

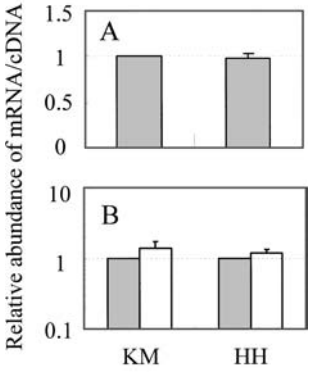

Fig. 5A, B Comparison of the relative abundance of cAPX transcripts between Kitamusume (*KM*) and Hokkaihadaka (*HH*) and between *GmAPX1* and *GmAPX2*. Total mRNA levels of *HH* were estimated by Northern-blot analysis as ratio to KM (**A**). Transcripts levels of *GmAPX1* were estimated by real-time PCR as the ratio to *GmAPX1* in each cultivar. Results were shown as means of three independent sample sets. *Bars* represent standard deviations

ing to the deficiency of APX1 (Fig. 4D, E). To determine the relative abundance of transcripts of the two genes encoding cAPX, real time PCR was performed. Although it revealed a slight difference in abundance between the transcripts of *GmAPX1* and *GmAPX2* (Fig. 5B) in both cultivars, neither of the cDNAs was so poor as could account for the deficiency of APX1 in Kitamusume. The deduced amino-acid sequence of *GmAPX2* (Accession No. AB082932) determined in Kitamusume was completely identical to that in Hokkaihadaka (Fig. 6). On the other hand, while there was no difference in the cDNA sequence of the coding region between *GmAPX1* of Hokkaihadaka (Accession No. AB082931) and *SOYAPx1*, we found two sequence variations in *GmAPX1* of Kitamusume (Accession No. AB082930). One is a single nucleotide polymorphism (SNP), which should generate substitution of an amino-acid residue (Phe26→Ile, Fig. 6), and the other was a single nucleotide deletion, which should create a substitution of four amino-acid residues and addition of seven amino-acid residues at the C-terminus due to a frame shift (Fig. 6). To clarify the relationship between this variant gene and the APX1 deficiency, genomic DNA from the F_2 plants derived from the cross between Kitamusume and Hokkaihadaka was amplified by PCR with primers specific to *GmAPX1* of Kitamusume and with those specific to *GmAPX1* of Hokkaihadaka. As shown in Fig. 7, only the Kitamusume type fragment was amplified in all 25 APX1-absent plants. Of 71 APX1-present plants, 28 plants carried only the Hokkaihadaka-type sequence and the others contained both types. The segregation agreed with the expected ratio of 1 Kitamusume type: 2 heterozygous: 1 Hokkaihadaka type ($0.5 < P < 0.75$).

Fig. 6 Deduced amino-acid sequences of soybean cAPX cDNA clones. *GMAPX1 – hh* represents the *GmAPX1* of Hokkaihadaka (Accession No. AB082931), which is completely identical to the *pSOYAP75* (cv Hobbit, Chatfield and Dalton 1993) and the *SOYAPx1* (cv Century, Caldwell et al. 1998). *GMAPX1 – km* represents the *GmAPX1* of Kitamusume (Accession No. AB082930). *GMAPX2* represents the *GmAPX2* (Accession No. AB082932) in the present study (cv Kitamusume and Hokkaihadaka). *SOYAPX2* represents *SOYAPx2* (cv Century, Caldwell et al. 1998)

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                        10         20         30         40         50
GMAPX1-hh.AMI     MGKSYPTVSA DYQKAVEKAK KKLRGFIAEK RCAPLMLRLA WHSAGTFDKG
GMAPX1-km.AMI     .......... .......... .....I.... .......... ..........
GMAPX2.AMI        .......... .......... .......... .......... ......Y.VS
SOYAPX2.AMI       .......... .......... .......... .......... ......Y.VS

                        60         70         80         90        100
GMAPX1-hh.AMI     TKTGGPFGTI KHPAELAHSA NNGLDIAVRL LEPLKAEFPI LSYADFYQLA
GMAPX1-km.AMI     .......... .......... .......... .......... ..........
GMAPX2.AMI        S......... ...S....G. .......... .......... ..........
SOYAPX2.AMI       S......... ...S....G. .......... .......... .T........

                       110        120        130        140        150
GMAPX1-hh.AMI     GVVAVEVTGG PEVPFHPGRE DKPEPPPEGR LPDATKGSDH LRDVFGKAMG
GMAPX1-km.AMI     .......... .......... .......... .......... ..........
GMAPX2.AMI        .......... .......... .......... .......... ..........
SOYAPX2.AMI       .......... .......... .......... .......... ..........

                       160        170        180        190        200
GMAPX1-hh.AMI     LTDQDIVALS GGHTIGAAHK ERSGFEGPWT SNPLIFDNSY FTELLSGEKE
GMAPX1-km.AMI     .......... .......... .......... .......... ..........
GMAPX2.AMI        .S.R...... .......... .......... .......... .K........
SOYAPX2.AMI       .S.R...... .......... .......... .......... .K........

                       210        220        230        240        250
GMAPX1-hh.AMI     GLLQLPSDKA LLSDPVFRPL VDKYAADEDA FFADYAEAHQ KLSELGF---
GMAPX1-km.AMI     .......... .......... .......... .......... ......LLMP
GMAPX2.AMI        .......... .......... .E...S.... .......... .......---
SOYAPX2.AMI       .......... .......... .E...S.... .......... .......---

                       260
GMAPX1-hh.AMI     ----ADA
GMAPX1-km.AMI     KQRLG.C
GMAPX2.AMI        ----.E.
SOYAPX2.AMI       ----.E.
```

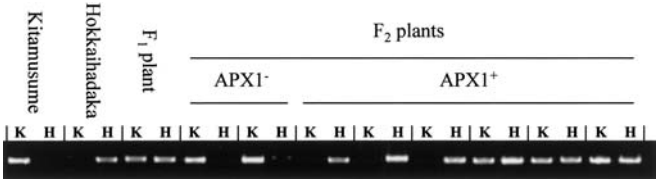

Fig. 7 Segregation of Kitamusume, Hokkaihadaka, and heterozygous types for *GmAPX1* in the F_2 family. *K* and *H* represent PCR products amplified with primers specific to *GmAPX1* of Kitamusume and to that of Hokkaihadaka, respectively. APX1$^-$ and APX1$^+$ represent APX1-absent and APX1-present F_2 individuals, respectively

Discussion

Combining the first step of the conventional breeder's approach and biochemical techniques, we detected polymorphism in the APX isozyme profile among soybean cultivars and lines with different levels of chilling tolerance. This finding may be of interest in several aspects. One is how the deficiency in an APX isozyme could associate with chilling tolerance, and another is how the expression of the gene is suppressed. Plant breeders may also pay their attention to whether it is possible to use the isozyme polymorphism as a genetic marker for selection of chilling-tolerant soybean plants and lines.

Evaluation of chilling tolerance

To estimate the degrees of chilling tolerance of soybean cultivars and lines, we employed field tests. In general, the results of field tests are most reliable in respect of the practical use, but they are always accompanied with relatively large experimental errors and annual fluctuations. There was indeed a little difference in the rank of cultivars and lines for CTI between 1997 and 1998. The results obtained in 1998 were in closer agreement with the usual ranking of cultivars for chilling tolerance (Sanbuichi 1979; TAES unpublished data) than those in 1997. This difference may result from the unusual temporary drastic low temperature in mid-August and the smaller plot area employed in 1997. Nevertheless, APX1-absent and APX1-present genotypes significantly differed in CTI in both years, suggesting that the genetic factor(s) associated with the APX1 deficiency has (have) a large effect on the expression of chilling tolerance. Moreover, the importance might be emphasized by the fact that the difference in CTI was more pronounced in 1998 than in 1997.

Genetic and molecular basis of APX1 deficiency

Caldwell et al. (1998) reported deficiency of the same isozyme as APX1 in the cultivar Roanoke. However, neither the genetic nor the molecular basis was presented. Therefore we conducted genetic analysis on the APX1 deficiency, which revealed that the APX1 deficiency is controlled by a single recessive gene. Furthermore, we performed the molecular analyses on cAPXs and their genes. The steady state levels of transcripts of the two cAPX genes were roughly equally abundant even in the APX1-absent genotype and there were no apparent differences in the size of transcripts between the two genotypes, suggesting the absence of deletion or insertion of large region at the loci of interest unlike CAT3 (Wadsworth and Scandalios 1990), *unifoliata* (Hofer et al. 1997) and dehydrin (Ismail et al. 1999) spontaneous mutants, which disable transcription or produce a smaller transcript. Therefore we examined whether SNPs and/or small insertions/deletions in the coding regions of the structural genes were involved, since such variations also cause naturally occurring loss-of-function mutations (Hofer et al. 1997; Ling et al. 1999; Yano et al. 2000). The complete coincidence of cDNA sequences in the protein-coding regions of *GmAPX2* between APX1-absent and APX1-present genotypes suggests no involvement of this gene in the APX1-deficiency. On the other hand, we found a SNP and a single nucleotide deletion in *GmAPX1* of the APX1-absent genotype. The complete correspondence of *GmAPX1* polymorphism with APX1 phenotypes in the F_2 family derived from the cross between the APX1-absent and the APX1-present genotypes suggests that the two *GmAPX1* genes are alleles at the *apx1* locus, and that the defective gene of the APX1-absent genotype confers the APX1 deficiency. The results also imply that APX1, the cAPX with higher mobility on nondenaturing gels, is the product of *GmAPX1* (*SOYAPx1*), which was unclear in the previous study (Caldwell et al. 1998). Considering the result of Western-blot analysis and the reduced activity of APX in APX1-deficient genotypes, the point mutation, which should cause a single amino-acid substitution, and/or the frame shift, which should result in substitution and addition of several amino-acid residues at the C-terminus of the coding region, may lead to the suppression of APX activity and the change in mobility on nondenaturing gels. It is also possible that the alterations of the nucleotide sequence and the amino-acid sequence cause inhibition of translation or activation of proteolysis.

Association of the APX deficiency with chilling tolerance

Many studies on ROS-scavenging enzymes have focused on changes or differences in the levels of enzyme activities. However, it is often ambiguous whether the increased activities are a reaction for protection in advance, or a result from the higher levels of stress, where genotypic differences are compared. For example, Pinhero et al. (1997) demonstrated that increased APX activities by chemical treatment could result in enhancement of chilling tolerance in a chilling-sensitive maize

cultivar, while a chilling-tolerant cultivar had a lower APX activity than the chilling-sensitive one, which might be brought about by higher antioxidant activities other than APX in the chilling-tolerant cultivar. We, therefore, paid our attention only to the presence or absence of isozymes, which may be a qualitative genetic trait and be little influenced by other factors. Although in some cases, cAPX is synthesized *de novo* after exposure to oxidative stress such as excess light (Karpinski et al. 1997), APX1 was not generated in APX1-deficient plants even under various stress conditions (Fig. 2), implying that the deficiency of the cAPX is not a 'result' of high antioxidant activities by enzymes and/or compounds other than the cAPX. This is also supported by the results of molecular analyses, which suggested the involvement of the defective gene in the APX1 deficiency.

Based on the result that the presence or absence of APX1 was consistent in all organs tested (Fig. 3), the genotype-dependent polymorphism of cAPX is likely to be conserved in tissues responsible for the genotypic differences in chilling tolerance, which are thought to include reproductive organs such as anthers (Goto and Yamamoto 1972; Kurosaki 2000) as well as vegetative parts (Sanbuichi 1979). Therefore it is possible that the deficiency or the reduced activity of cAPX itself is involved in the enhancement of chilling tolerance. A possible explanation is that excessive levels of cAPX in APX1-present plants may scavenge "too much" of H_2O_2 in cytosols, hindering the expression of the defense genes, which could be induced by H_2O_2 (Desikan et al. 2001). This hypothesis might be supported by the recent finding that too-high levels of APX could reduce tolerance to oxidative stresses (Shi et al. 2001).

The introduction of exogenous genes and the enhancement of endogenous genes encoding antioxidant enzymes have reportedly resulted in increased chilling tolerance (Gupta et al. 1993; Prasad 1997; Roxas et al. 1997; Van Breusegem et al. 1999a, b; Sato et al. 2001). Therefore we had presumed that chilling-tolerant soybean cultivars would have, if any, additional isoforms of antioxidant enzymes. However, the present study showed that the genotypes lacking a cAPX isozyme exhibit relatively high chilling tolerance. Our finding may suggest the complexity in the mechanism underlying chilling tolerance, especially when evaluated by seed-yielding capacity in the field. This is not surprising considering that in the most previous studies cited, only foliar damage in strictly controlled conditions has been used as an index for chilling tolerance.

However, we do not exclude the possibility of indirect association of APX deficiency with chilling-tolerance. The *apx1* locus could be closely linked to QTLs for chilling-tolerance, since the APX1 deficiency is likely to be derived from a relatively small-scale germplasm (data not shown).

Although it remains to be elucidated how the deficiency of APX1 associates with chilling-tolerance, we believe that our findings are informative for further genetical and physiological studies on soybean chilling-tolerance. For instance, the isozyme and DNA polymorphisms observed can be used as a genetic marker in QTL analysis of chilling-tolerance, and they may further be used as a marker for selection of chilling-tolerant genotypes. It should also be noted that there is no linkage between the *apx1* locus and the previously reported loci associated with chilling-tolerance, suggesting the role of a newly identified gene for chilling-tolerance of soybean.

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